EXHIBIT D

PAIN

Pain 71 (1997) 313–322

Antinociception induced by civamide, an orally active capsaicin analogue

Xiao-Ying Hua*, Ping Chen, Jai-hyun Hwang, Tony L. Yaksh

Anesthesia Research Laboratory, Department of Anesthesiology, 0818, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093–0818, USA

Received 10 July 1996; revised version received 6 February 1997; accepted 27 February 1997

Abstract

The antinociceptive effects of a novel capsaicin analogue, civamide (cis-8-methyl-*N*-vanillyl-6-nonenamide), given orally to adult rats were examined. In the formalin test, civamide significantly suppressed the flinch response, particularly phase 2, in a dose-dependent fashion (20–200 mg/kg). This inhibitory effect started 1 h after application, and was maintained for 4–7 days. A competitive capsaicin antagonist, capsazepine (15 mg/kg, s.c.), reversed the antinociceptive action of civamide (200 mg/kg) on the formalin test when it was given either 5 min or 55 min after oral civamide delivery. In contrast, capsazepine delivered 2 days after civamide had no effect upon the depressed formalin response. Civamide produced a significant increase in the response latency on the thermal paw withdrawal test, which persisted for 2–3 days. Civamide produced a modest, but statistically significant, reversal of low tactile thresholds otherwise observed in the Chung neuropathic rats. Morbidity (approximately 10%) was observed which was secondary to bronchial constriction occurring with gastric reflux. Civamide at the doses given did not produce motor dysfunction. Neither calcitonin gene-related peptide (CGRP) nor substance P (SP) concentrations in dorsal or ventral spinal cord were altered by civamide (200 mg/kg) up to 5 days, whereas CGRP, but not SP, in dorsal root ganglia (DRG) and sciatic nerves was modestly reduced at 1 day after the delivery. These data suggest that an orally bioavailable capsaicin analogue, civamide, possessed analgesic activity with respect to several noxious stimuli, including inflammation-induced hyperalgesia, noxious thermal stimulation and nerve injury-induced tactile allodynia. The rapid onset and lack of change in the peptide levels in dorsal spinal cord suggests that the analgesic action of civamide is primarily a result of desensitization at the afferent terminals. The antinociception of civamide is probably mediated by at least two mechanisms: (i) an acute receptor occupancy dependent effect; and (ii) a persistent and receptor independent effect which is initiated by the acute exposure to the drug.

Keywords: Capsaicin; Analgesia; Desensitization; Sensory nerves; Substance P; Calcitonin gene-related peptide

1. Introduction

Exposure to capsaicin results in the activation, desensitization, and under certain conditions, the destruction of lightly myelinated or unmyelinated primary afferents fibers. (Holzer, 1991). The latter two effects are believed to account for the antinociceptive effects of systemic capsaicin, as observed in a number of behavioral models (Yaksh et al., 1979; Hayes and Tyers, 1980; Hayes et al., 1984; Jhamandas et al., 1984; Dickenson et al., 1990a; Perkins and Campbell, 1992). While the effect of capsaicin may be in part attributable to impairment of afferent function at the peripheral terminals, there is evidence supporting the hypothesis that the antinociceptive effects may result from its action at the spinal terminals of the afferents (Yaksh et al., 1979; Jhamandas et al., 1984; Dickenson et al., 1990a).

The mechanism of this drug effect has been a subject of considerable interest. Based on structure-activity studies (Szolcsanyi and Jancso-Gabor, 1975, 1976; Hayes et al., 1984; Jhamandas et al., 1984), binding studies (Szallasi and Blumberg, 1990) and the development of specific antagonists, such as capsazepine (Bevan et al., 1991; Dray et al., 1991), it seems certain that the effects of the capsaicinoids are mediated by an interaction with a specific membrane receptor (Szallasi and Blumberg, 1990; Wood et al., 1990). The acute occupation leads to the activation and

* Corresponding author. Tel.: +1 619 5435446; fax: +1 619 5456070.

0304-3959/97/$17.00
PII S0304-3959(97)00003-1

desensitization of the terminal with which the receptor is associated. The acute desensitization of the terminal is believed to account for the attenuation of the behavioral responses otherwise evoked by afferent input. This action, leading to a persistent, but reversible, antinociception is believed to be different from the antinociception observed in animals treated neonatally with capsaicin. Neonatal capsaicin treatment causes a permanent degeneration of unmyelinated afferent nerves, and a continued loss of thermal and chemical nociception in adult animals (Jancso et al., 1977; Holzer, 1991).

An important limitation of capsaicin is the powerful stimulatory effect of this drug (Holzer, 1991). Although the desensitizing properties of these agents might co-vary with the stimulatory activity (Szolcsanyi and Jancso-Gabor, 1975, 1976; Hayes et al., 1984; Jhamandas et al., 1984), recent studies have suggested that these properties may be dissociated. Dickenson et al. (1990b) reported that intradermal injection of a capsaicin analogue olvanil, which is orally active (Brand et al., 1987), does not activate C-fibers, but does attenuate C-fiber evoked responses.

In the present study, we carried out experiments to investigate possible antinociceptive effects of a second orally bioavailable capsaicin analogue, civamide (cis-8-methyl-*N*-vanillyl-6-nonenamide). We attempted to define: (i) the effect of oral delivery of civamide on three models of nociception: formalin test (an inflammatory pain model) (Wheeler-Aceto et al., 1990; Malmberg and Yaksh, 1992), thermal paw withdrawal test (an acute pain model), and the Chung model (a nerve injury model yielding tactile allodynia) (Kim and Chung, 1992; Chaplan et al., 1994); (ii) the capsaicinoid receptor pharmacology of the antinociceptive effects of civamide using a competitive capsaicin antagonist capsazepine; and (iii) the effect of oral civamide on calcitonin gene-related peptide (CGRP) and substance P (SP) contents in sensory nerves.

2. Methods

2.1. Animals

Male rats (Harlan Sprague Dawley, Indianapolis, 320–360 g for thermal paw withdrawal and formalin tests, and 120–140 g for Chung model) were used in accordance with protocols approved by the Animal Care Committee of the University of California. The animals were maintained in a group colony on an ad libitum diet and on a 12-h day/12-h night cycle.

2.2. Testing

2.2.1. Formalin test

To perform the formalin test, rats were placed in a plexiglas box connected to a vaporizer and briefly anesthetized with halothane (3%). After 2–3 min there was a temporary loss of spontaneous movement with preservation of the deep spontaneous respiration, and blink and pinnae reflexes. The animal was then quickly removed, and 50 μl of 5% formalin solution was injected subcutaneously into the dorsal surface of the right hind paw with a 30-gauge needle. The rats were then individually placed in an open plexiglas chamber for observation, and within a maximum interval of 1–2 min the animal displayed recovery from anesthesia with spontaneous activity and normal motor function. A mirror was placed on the opposite side of the plexiglas chamber for the unhindered observation of the formalin injected paw. Pain behavior was quantified by counting the flinching/shaking of the injected paw. Animals were observed individually and the flinches counted for 1-min periods at 1–2 min, 5–6 min and at 5-min intervals for 60 min. Two phases of spontaneous flinching behavior were observed as previously described (Wheeler-Aceto et al., 1990): phase 1 started immediately after formalin injection and lasted through the second observation interval (5–6 min), followed by phase 2 which began after 10 min with a maximum response typically observed around 30–40 min after the formalin injection. After the observation period of 1 h, animals were immediately sacrificed with an overdose of barbiturate mixture (Beuthanasia, 50 mg/kg, i.p.) (Malmberg and Yaksh, 1992). Thus an individual rat was used for one experiment only in formalin test.

2.2.2. Thermal paw withdrawal

Thermal paw withdrawal (TPW) was measured by placing the animals in plexiglas cages (9 × 22 × 25 cm), on a modified Hargreaves Box (UARDG, Department of Anesthesiology, University of California, San Diego, La Jolla, CA). This device consisted of a glass surface with a maintained surface temperature of 30°C. A stimulus lamp was focused on the plantar surface of the paw with an attached mirror which permitted visualization of the under surface. An abrupt withdrawal of the hind paw subsequent to the stimulus was sensed by photodiodes, and this served to terminate the stimulus and stop the timer. In order to avoid tissue damage, failure to respond in 20 s terminated the test (cut-off time) and this latency (20 s), was assigned. After a 20-min adaptation period, the first measurement was undertaken in both hind paws. The response latencies were averaged and counted as baseline score (time 0) (Hargreaves et al., 1988). Each individual rat (vehicle or civamide with a single dose) employed for TPW test was used for a full time-course study (60 min, days 1, 2, 3 and 4 after civamide application).

2.2.3. Chung model

For creating the neuropathic preparation (Chung model), the surgical procedure previously described (Kim and Chung, 1992) was performed to induce an allodynic state. Briefly, under halothane anesthesia, the left L-5 and L-6 spinal nerves were isolated adjacent to the vertebral column and ligated tightly with No. 6–0 silk suture just distal to the

dorsal root ganglion, proximal to the formation of the sciatic nerve. The rats usually developed tactile allodynia by post-operative day 2, and the allodynia was maintained for at least 20 days (Chaplan et al., 1994). For testing, the rats were placed in plastic cages with a wire mesh bottom, divided into individual compartments of 5 × 6 × 9 in. Behavioral accommodation was allowed for approximately 15 min until cage exploration and major grooming activities ceased. Testing the tactile threshold required evoking withdrawal of the stimulated paw. Von Frey hairs were applied to the paw of the ligated nerve (left). The area tested was the mid-plantar left hind paw, in the sciatic nerve distribution, avoiding the tori (foot pads). The 50% withdrawal threshold was determined using the up-down method (Chaplan et al., 1994) beginning with the 2.0-g hair, in the middle of the series of eight von Frey hairs with logarithmically incremental stiffness ranging from 0.41 to 15.1 g (Nos. 3.61 to 5.18). Each hair was pressed perpendicularly against the paw with sufficient force to cause slight bending, and held for approximately 6–8 s. A positive response was recorded if the paw was sharply withdrawn. Based on observations on normal, unoperated rats and sham-operated rats, the cut-off of the 15.1-g hair was selected as the upper limit for testing. The rats which developed tactile allodynia with a 50% threshold of 4.0 g or less were used in the study (Chaplan et al., 1994). The animals were recovered for 7 days after the surgery. An individual Chung rat (vehicle or a single dose of civamide) was used for a full time-course study (60 min, days 1, 2, 3 and 4 after civamide delivery).

2.3. Tissue collection and radioimmunoassays

2.3.1. Tissue collection

On the second day after receiving vehicle ($n = 6$), or civamide 200 mg/kg ($n = 6$), 12 rats were sacrificed with phenobarbital (60 mg/kg i.p.). Six lumbar dorsal root ganglia (DRG), 1-cm segment of dorsal and ventral spinal cord (L2–L4), sciatic nerve, vagus nerve and trachea were dissected out. The tissues were extracted in 1 ml 0.1 M HCl in a boiling water bath. Following 10 min of boiling, the tissues were homogenized using a Polytron device. The homogenates were subsequently centrifuged and the supernates collected, lyophilized and subjected to radioimmunoassay (RIA), to measure CGRP and SP. The peptide levels in dorsal spinal cords were analyzed in another group of rats on the fifth day after receiving civamide 200 mg/kg ($n = 5$) or vehicle ($n = 6$).

2.3.2. CGRP-RIA

CGRP levels in spinal cord tissue extracts were measured by RIA using CGRP antibody G 2027 (1:10 500) and [I^{125}]Tyr^0 CGRP tracer. G 2027 was developed and produced in our laboratory. The absolute sensitivity is 3 fmol/assay tube. G 2027 cross-reacts 100% with rat CGRP-β, human CGRP-α and human CGRP-β, but does not recognize NKA, SP, calcitonin, $CGRP_{29-37}$, or cholecystokinin-8 (<1% at 10–100 μg/ml). CGRP-immunoreactivity has been characterized by high performance liquid chromatography. Immunoreactive material detected by antibody G 2027 was eluted in a single peak at the same fraction as synthetic rat CGRP-β (Hastings and Hua, 1995).

2.3.3. SP-RIA

SP-immunoreactivity was assayed using rabbit antibody No. 4893 (1:120 000), and [I^{125}]Tyr^8 SP as the tracer (Go and Yaksh, 1987). The absolute sensitivity of the assay is 1.5 fmol/assay tube with inter- and intra-assay variation 4 and 11%. SP antibody No. 4893 does not cross-react to NKA (<1% at 700 pmol/tube), CCK, VIP and bombesin (<0.1%). All assays were carried out in duplicate, and non-specific binding and blanks were also assessed. Standard SP and rat CGRP-β were purchased from Peninsula.

2.4. Drug treatment

Oral civamide (20 mg, 60 mg and 200 mg/kg) delivery was accomplished by the use of blunt 16-gauge gavage needles (Popper and Sons, Inc.). Stock solution of civamide 10%, was used for the dose of 200 mg/kg. Further dilutions of stock civamide were made with the vehicle propylene glycol (Ruger, Irvington, NJ) to 3% and 1% of civamide solution, which were used for doses of 60 mg/kg and 20 mg/kg, respectively. Drug/vehicle was given in volume of 0.6–0.7 ml. TPW test and von Frey hair test were carried at time points of 60 min, day 1 (2nd day), day 2 (3rd day), day 3 (4th day) and day 4 (5th day), post-drug/vehicle delivery; and the formalin test was carried at 60 min and day 2 (3rd day), day 4 (5th day) and day 7 (8th day).

2.5. Statistical analysis

The TPW response latency data (in seconds) and the tactile threshold (in grams) were converted to %MPE (maximal possible response), calculated as:

$$\%\text{MPE} = [\text{Post-application response} - \text{pre-application response}]/[\text{Maximal possible response} - \text{pre-application response}] \times 100$$

where the maximal possible responses were 20 s in TPW test and 15 g in paw withdrawal threshold in von Frey hair test.

For the formalin test, time-response data are presented as the number of flinches for the intervals of 1–2 min, 5–6 min and at 5-min intervals after that up to 60 min. Dose-response curves are presented as the sum of flinches for the observation periods, i.e., total number of flinches of phase 1 (0–9 min), and phase 2 (10–60 min), respectively.

All of the data are presented as mean ± SEM, and statistical significance was calculated using the unpaired t-test and one-way analysis of variance with multiple comparison for independent measurement followed by a Fisher test.

Differences were considered to be significant when the critical value reached a level of $P < 0.05$.

3. Results

3.1. General observations

Rats receiving civamide at a dose 20 mg/kg, p.o. appeared normal in all respects except that they displayed evident salivation by 1–5 min after gavage and this salivation continued until around 30–40 min. Salivation was profound at the higher doses (60 and 200 mg/kg) (Table 1). Some animals at the highest dose displayed an elongated body posture with the abdomen pressed to the floor, a behavior which resembled the 'writhing' response induced by interperitoneal irritants (Schmauss and Yaksh, 1983). This occurred in animals within 10–20 min after receiving the 60- and 200-mg/kg doses. Salivation and posturing symptoms ceased within 60 min and were not seen again in the remaining observation period. We did not observe motor weakness or dysfunction in civamide-treated animals. All rats during the experimental period showed symmetrical ambulation with normal elevation and placing of either hind paw in a co-ordinated fashion. Hind limb strength was evidenced by the ability to rear and groom with a symmetrical posture. Pinna reflex and cornea reflex in civamide-treated animals were routinely present after all doses (Table 1).

Death was observed in 6/43 rats treated with civamide 200 mg/kg, 2/20 with 60 mg/kg, 0/21 with 20 mg/kg and 0/31 with vehicle. Morbidity, when it occurred, happened immediately after the gavage of civamide. In these cases, we believe that gastric reflux had occurred at the time the oral probe was withdrawn. We consider that death was secondary to bronchial constriction. In the absence of acute respiratory distress, rats displayed normal motor function and behavior. There was no loss in body weight 4 days after civamide applied at any of the three doses (Table 2).

Table 1
Effects of oral civamide on pinna reflex, cornea reflex and salivation

Treatment	Pinna reflex (n)	Cornea reflex (n)	Salivation (n)
Vehicle	Normal (8)	Normal (8)	None (8)
Civ 20 mg/kg	Normal (7)	Normal (7)	None (1), + (5), ++ (1)
Civ 60 mg/kg	Normal (6)	Normal (6)	+ (4), ++ (2)
Civ 200 mg/kg	Normal (5)	Normal (5)	+ (1), ++ (4)

Civ, civamide; +, moderate salivation; ++, severe salivation.

Table 2
Body weight of rats 4 days after receiving civamide/vehicle

Treatment	N	Body weight (g)	
		Before	After
Vehicle	8	349 ± 5	348 ± 6
Civ 20 mg/kg	7	349 ± 3	345 ± 3
Civ 60 mg/kg	6	324 ± 7	324 ± 7
Civ 200 mg/kg	5	327 ± 2	332 ± 2

Civ, civamide.

3.2. Formalin test

As previously noted, formalin injected into the paw caused flinching behavior which was characterized by two phases. Phase 1 started with initial intense flinches occurring in 1–2 min, followed by a rapid decline in 5–6 min. Phase 2 began after 20–25 min with a maximum response typically observed around 30–45 min after the formalin injection (Malmberg and Yaksh, 1992).

3.2.1. Civamide treatment

In vehicle-treated rats, the time-course of flinch activity was the same as that previously reported in untreated rats. Thus, the total number of flinches in phase 1 (1–9 min), was 23 ± 2, and in phase 2 (10–60 min) 133 ± 16 in vehicle-treated rats ($n = 17$) (Figs. 1 and 2).

Sixty min after application of civamide 200 mg/kg, a significant reduction of flinches in both phase 1 (16 ± 2, 70% of the vehicle group, $n = 13$, $P < 0.05$) and phase 2 (54 ± 7 flinches/min, 41% of control, $n = 13$, $P < 0.01$) was observed (Fig. 1A and Fig. 3), and this effect remained on day 2 (phase 1: 14 ± 4 and phase 2: 39 ± 13 flinches/min, 29–61% of the vehicle group, $n = 6$, $P < 0.01$, Fig. 1B and Fig. 3). The inhibitory effect of civamide on phase 1 was diminished on day 7 (24 ± 2, 104% of the vehicle group, $n = 6$), whereas the effect on phase 2 (72 ± 23, 54% of the vehicle group, $n = 6$, $P < 0.05$) was still present, though there was a tendency to return to baseline level (Fig. 3). Overall, civamide demonstrated dose-dependent effect on phase 2 flinches ($P < 0.05$, Fig. 2).

3.2.2. Civamide + capsazepine treatment.

In other experiments, we examined the antagonistic effect of capsazepine on the civamide-induced analgesic action on the formalin test at two time points: 1 h and 48 h after civamide administration.

3.2.2.1. Formalin test 1 h after civamide. Civamide was given at t (time) = 0, and the formalin test was initiated at $t = 1$ h. Capsazepine (15 mg/kg, s.c.) (Perkins and Campbell, 1992) was applied either 5 min ($n = 8$), or 55 min ($n = 5$) after civamide delivery. Both treatments significantly reversed the antinociceptive action of civamide as measured on the formalin test, phase 2 behavior at 1 h (Fig. 4).

3.2.2.2. Formalin test 2 days after civamide. Civamide was given at $t = 0$, and the formalin test was initiated at $t = 48$ h. When capsazepine (15 mg/kg, s.c.) was applied 5 min

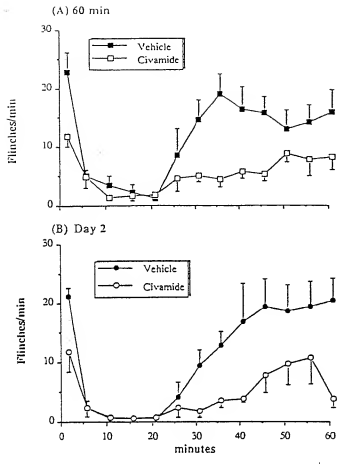


Fig. 1. The time-course effect of civamide 200 mg/kg, p.o. on the flinch response (flinches/min) evoked by injection of 50 μl of 5% formalin into the paw at 60 min (A) and at the third day (day 2) (B) after civamide/vehicle delivery. The data are presented as mean ± SEM of 5–6 animals in each group.

after civamide, the antinociception induced by civamide at $t = 48$ h was prevented ($n = 5$). In contrast, the analgesic effect of civamide at $t = 48$ h was not altered by capsazepine when the antagonist was given 5 min prior to the formalin injection (e.g., 48 h after civamide) ($n = 6$; Fig. 5).

3.3. Thermal nociception

The paw withdrawal latency to thermal stimulation in vehicle-treated rats (control group) was 8.6 ± 0.5 s ($n = 8$). Oral civamide at the three doses did not change paw withdrawal latency 1 h after administration of the drug. One and 2 days after being given civamide (200 mg/kg), a moderate, but statistically significant increase in paw withdrawal latency was observed ($P < 0.05$). The effect gradually declined towards the pre-drug level by day 3 and day 4. As indicated, the effect of civamide on the TPW test was dose-dependent (20–200 mg/kg) on day 2 ($P < 0.05$) ($n = 5$–8; Fig. 6).

3.4. Chung model

Following nerve ligation, all the rats displayed a significant reduction in the mechanical stimulation threshold at which a withdrawal response of the injured hind paw to the von Frey hair stimulation was observed. Thresholds after surgery were uniformly between 1 and 3 g compared with 15 g or greater in contralateral, non-operated paw. Oral vehicle ($n = 4$) had no effect upon the tactile threshold, while civamide at 200 mg/kg ($n = 11$) displayed a modest but statistically significant anti-allodynic activity on the second day ($P < 0.05$, one-way ANOVA; Fig. 7). The elevated mechanical threshold returned to baseline level in 4–5 days (Fig. 7A). The anti-allodynic effect of civamide on the Chung model was dose-dependent ($P < 0.05$, $n = 6$-11; Fig. 7B).

3.5. Peptide levels in central and peripheral tissues

There was no significant change in SP content in DRG, ventral/dorsal spinal cord, sciatic nerve or vagus nerve 1 day

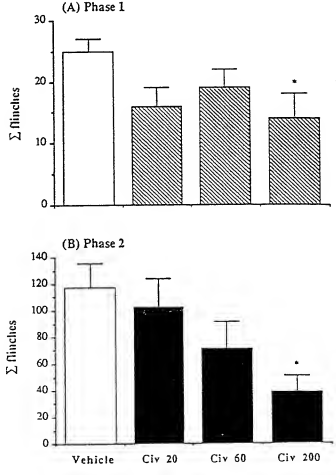


Fig. 2. Summary of the dose-response effect of oral civamide (Civ 20, 60 and 200 mg/kg) on the flinch response (S flinches: total flinches/phase) in the formalin test, phase 1 (1–9 min) (A) and phase 2 (10–60 min) (B), at the third day (day 2) after civamide/vehicle administration. $n = 5$–10; *$P < 0.05$, one-way ANOVA.

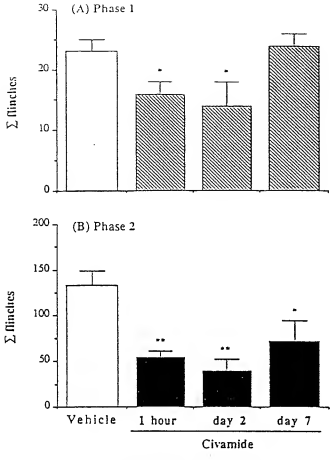


Fig. 3. The recovery time-course of the effect of civamide 200 mg/kg p.o. on the formalin test, phase 1 (A) and phase 2 (B) at 1 h ($n = 13$), at the third day (day 2, $n = 6$) and at the eighth day (day 7, $n = 6$) after civamide delivery. The vehicle group includes results from the tests carried out on day 2 and day 7. $N = 17$; $^*P < 0.05$, $^{**}P < 0.01$, one-way ANOVA.

after oral application of civamide (200 mg/kg) (Table 3). In contrast, CGRP in DRG neurons and sciatic nerves was reduced significantly by civamide treatment although there was no significant change in ventral or dorsal spinal cord (Table 4). Both SP and CGRP contents in the trachea were significantly enhanced (Table 4). Neither SP nor CGRP in dorsal spinal cord was attenuated even 5 days after civamide administration (vehicle versus civamide: SP 6.9 ± 0.8 versus 7.3 ± 0.6 and CGRP 16.3 ± 0.8 versus 16.3 ± 1.6 pmol/mg protein, $n = 5$-6).

4. Discussion

The present studies provide evidence that civamide is an orally active agent, which displays persistent, but reversible, antinociceptive activities on several noxious stimuli, including inflammation-induced hyperalgesia, noxious thermal stimulation and nerve injury-induced tactile allodynia.

4.1. Formalin test

The effect on the formalin test was marked. Civamide significantly blocked nociceptive behavior in both phase 1 and phase 2, and the effect was long-lasting (4–7 days). The formalin test consisted of two phases of flinching behavior. The first phase is believed to reflect the acute afferent drive evoked by the initial action of the formalin. Previous work has shown that the afferent input occurring during the first phase results in the initiation of a facilitated state of afferent processing (Dickenson, 1990). This facilitated state of processing, in conjunction with the modest afferent input, is believed to account for the pronounced behavior in the second phase. Small afferent C-fibers are known to play an important role in formalin-induced pain behavior, since it is evident that neonatal capsaicin treatment produces a significant reduction of response in the formalin test (Nagy and van der Kooy, 1983; Hara et al., 1984). The injection of formalin into the paw results in an increase in SP release in dorsal spinal cord (Kantner et al., 1985; Kuraishi et al., 1989). Spinal pretreatment with the NK1 receptor antagonist CP-96,345 has modest effects upon phase 1, but significantly reduces phase 2 (Yamamoto and Yaksh, 1991).

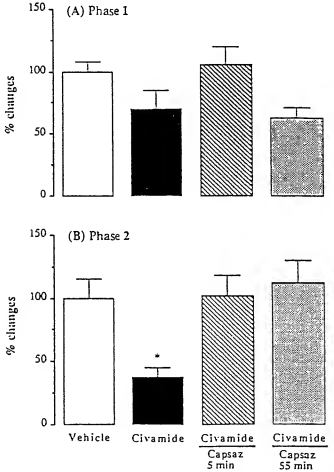


Fig. 4. Effects of the capsaicin antagonist capsazepine (Capsaz) on civamide given 1 h before formalin injection, phase 1 (A) and phase 2 (B). Capsazepine was given either 5 min after ($n = 8$) or 55 min after ($n = 5$) civamide administration. The data are presented as % changes of the vehicle group (Σ flinches: phase 1, 22 ± 2; phase 2, 139 ± 21; $n = 12$). Civamide group: $n = 8$. $^*P < 0.05$, one-way ANOVA. No statistical significance between the groups in phase 1.

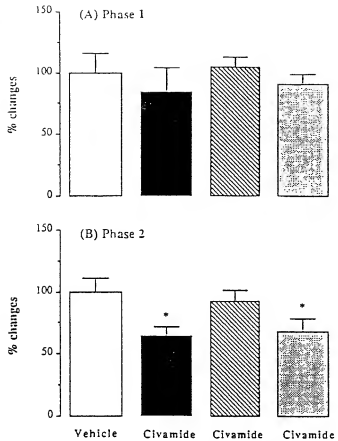


Fig. 5. Effects of the capsaicin antagonist capsazepine (Capsaz) on civamide given 2 days before formalin test, phase 1 (A) and phase 2 (B). Capsazepine was given either 5 min ($n = 5$) after or 2 days after but 5 min prior ($n = 6$) to civamide administration. The data are presented as % changes of the vehicle group (Σ flinches: phase 1, 21 ± 3, phase 2, 210 ± 23; $n = 5$). Civamide group: $n = 5$. *$P < 0.05$, one-way ANOVA. No statistical significance between the groups in phase 1.

This observation has been taken to indicate that while spinal NK1 receptors have little effect upon the behavior in phase 1, they mediate the processes which lead to the facilitated components of phase 2. Based on the presumed action of capsaicinoid agents on the small afferent, it is likely that the antinociceptive effect of civamide observed in the formalin test is attributable to the interruption of small afferent (tachykinin/CGRP) transmission, perhaps secondary to an acute desensitization at the afferent terminal (see discussion below).

4.2. Acute thermal nociception

Oral civamide in tolerable doses was able to produce a protracted and statistically significant increase in the thermal response latency. This effect evolved within an interval of greater than 1 h and less than 24 h and lasted for 2–3 days. This effect is consistent with previous work with capsaicin which has indicated that can produce a thermal antinociception (Fitzgerald and Woolf, 1982; Gamse, 1982; Perkins and Campbell, 1992), although some studies show no change or even reduction in thermal thresholds (Hayes and Tyers, 1980). Since the behavioral response in phase 1 of the formalin test was significantly inhibited by civamide, it appears that this agent is able to block acute nociception. Accordingly, this action is consistent with an effect upon C-polymodal nociceptors.

4.3. Neuropathic pain

The inhibitory effect of civamide upon the Chung model was moderate, but statistically significant. There was no evidence of non-specific effects upon behavior (i.e., motor weakness), and it is believed that the changes indeed reflected a real effect upon this neuropathic pain state. Tactile allodynia in the Chung model is believed to depend primarily upon the activity of low-threshold mechanoceptors and is sympathetically dependent (Kim et al., 1993). The responses in the Chung model are poorly affected by spinal opiates, but they can be reversed by reduction in sympathetic tone (Yaksh et al., 1995). Some studies in other neuropathic pain models, however, have shown that the thermal hyperalgesia produced by sciatic nerve injury, was prevented by neonatal capsaicin treatment (Shir and Seltzer, 1990; Meller et al., 1992). The findings from a recent study carried out using a new model of neuropathic pain in rats, in which partial nerves innervating the tail are transected (Kim et al., 1995), have suggested that the capsaicin-sensitive sensory nerves play a critical role in the development of both mechanical and thermal allodynia. It has been suggested that Aδ and C-fibers may participate in a

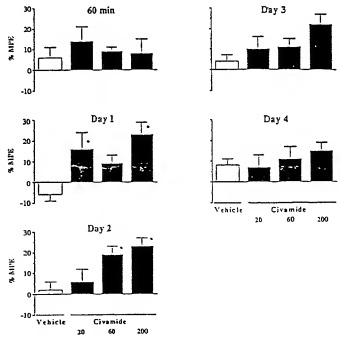


Fig. 6. The effect of civamide (20, 60 and 200 mg/kg, p.o.) on thermal paw withdrawal test, at 60 min, second day (day 1), third day (day 2), fourth day (day 3) and fifth day (day 4) after civamide/vehicle delivery. $N = 5$–8; *$P < 0.05$, one-way ANOVA.

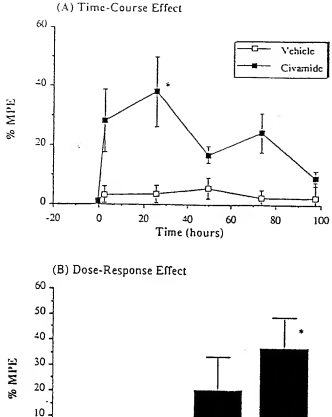


Fig. 7. Summary of (A) the time-course effect of civamide 200 mg/kg, and (B) the dose-response effect of civamide (20, 60, and 200 mg/kg, p.o.) measured on the second day after drug application on the Chung model. The data are presented as mean ± SEM of 5–11 animals in each group. *$P < 0.05$, one-way ANOVA.

central sensitization produced by nerve injury (Kim et al., 1995). The effects of civamide on the Chung model is apparently in agreement with this hypothesis. A significant improvement in pain score has been demonstrated in clinical trials in neuropathic pain patients with topically or locally administered capsaicin (Winter et al., 1995). Some patients, however, with chronic post-herpetic neuralgia appear resistant to the capsaicin treatment (Watson et al., 1993).

4.4. Mechanisms of drug action

4.4.1. Civamide and the capsaicinoid receptor

Evidence supporting the existence of a specific membrane receptor for capsaicin has been based on: (i) studies on structure-activity relationships employing a variety of capsaicin derivatives (Szolcsanyi and Jancso-Gabor, 1975, 1976; Hayes et al., 1984; Jhamandas et al., 1984) and a capsaicin-like photoaffinity probe (James et al., 1988); (ii) identification of specific capsaicin binding sites by using the

Table 3

SP-immunoreactivity (pmol/mg protein) in rat tissue 1 day after oral application of civamide (200 mg/kg) or vehicle (equal volume) ($n = 6$ for each group)

Tissue	Vehicle	Civamide	P-value
DRG	0.27 ± 0.04	0.26 ± 0.02	NS
V-cord[a]	1.17 ± 0.13	1.40 ± 0.13	NS
D-cord[b]	4.72 ± 0.77	6.68 ± 0.95	NS
N. sciatic	0.17 ± 0.02	0.14 ± 0.02	NS
N. vagus	0.12 ± 0.01	0.14 ± 0.02	NS
Trachea[c]	1.23 ± 0.19	2.23 ± 0.47	<0.05

[a]Ventral spinal cord.
[b]Dorsal spinal cord.
[c]SP in trachea is represented as pmol/mg wet tissue.

ultrapotent capsaicin analogue, resiniferatoxin, as a binding ligand (Szallasi and Blumberg, 1990); and (iii) development of a competitive antagonist, capsazepine, which is highly selective in its ability to block capsaicin-induced effects (Bevan et al., 1991; Dray et al., 1991; Perkins and Campbell, 1992). It has been demonstrated that capsazepine antagonizes capsaicin-evoked depolarization in cultured DRG cells (Bevan et al., 1991), and capsaicin-induced neuronal excitation in a spinal cord-tail preparation and a saphenous nerve-skin preparation (Dray et al., 1991). While lacking antinociceptive and anti-inflammatory activity of its own, capsazepine reverses the analgesic action of capsaicin in both acute and chronic pain models (Perkins and Campbell, 1992). The fact that the antinociceptive effect of civamide was reversed by capsazepine in the present study at a similar dose range as employed previously (Perkins and Campbell, 1992), suggests that the effect of civamide is also mediated via an action on capsaicinoid receptors.

4.4.2. Spinal depletion and terminal desensitization

The mechanism whereby capsaicinoid receptor occupancy by civamide induces the persistent (2–4 days) antinociceptive action is not clear. Early studies, particularly with neonatal animals, emphasized the ability of these agents to deplete the terminal stores of transmitter peptides

Table 4

CGRP immunoreactivity (pmol/mg protein) in rat tissue 1 day after oral application of civamide (200 mg/kg) or vehicle (equal volume) ($n = 6$ for each group)

Tissue	Vehicle	Civamide	P-value
DRG	1.92 ± 0.16	1.47 ± 0.07	<0.05
V-cord[a]	0.39 ± 0.09	0.24 ± 0.04	NS
D-cord[b]	12.1 ± 1.98	10.33 ± 1.42	NS
N. sciatic	1.18 ± 0.12	0.80 ± 0.07	<0.05
N. vagus	0.31 ± 0.15	0.37 ± 0.03	NS
Trachea[c]	10.5 ± 2.46	17.83 ± 1.66	<0.05

[a]Ventral spinal cord.
[b]Dorsal spinal cord.
[c]CGRP in trachea is represented as pmol/mg wet tissue.

and to cause frank neurolysis (Jancso et al., 1977; Nagy and van der Kooy, 1983). Thus, the antinociceptive activity of systemic capsaicin treatment (neonatal or adult) is normally associated with a decrease in SP/CGRP contents in spinal dorsal horn (Holzer, 1991). A single injection of capsaicin (50–125 mg/kg, s.c.) in adult rats caused a significant reduction of SP (30–50% of control) in dorsal spinal cord within 4 days (Ganise et al., 1981). In contrast, neither SP nor CGRP in the dorsal spinal cord were changed by civamide at intervals of up to 5 days. There was a moderate but significant reduction of CGRP level in DRG and sciatic nerve at 1 day after civamide. While histopathology has not been reported, these results would not support a prominent role for a neurotoxic action in explaining the effects of civamide.

Several observations suggest that in addition to a neurotoxic action, the effects observed on application of civamide may indicate a desensitization phenomenon. First, the onset of civamide action was rapid. On the formalin test, the effect was observed by 1 h after drug delivery, while the effects on acute thermal nociception and tactile allodynia started at some time after 60 min and within 24 h. Second, treatment with the capsaicinoid antagonist capsazepine at an interval of up to 55 min after the civamide had been delivered prevented the suppression of the formalin response. Significantly, this early treatment prevented the manifestation of the formalin suppression otherwise observed 2 days after civamide. In contrast, the antagonist treatment 2 days after civamide was without effect, i.e., the response to formalin injection remained suppressed. These observations are extremely useful in generating hypotheses as to the actions of this agent. Capsazepine given 55 min after civamide treatment prevented the initial phase, and this suggests that the process of generating the prolonged antinociception in this model requires some minimum exposure time. Conversely, the property of the acute antagonism (e.g., approximately 15–20 min before phase 2) indicates that the early effects of civamide are rapidly reversible, or in fact require a continued occupancy of the capsaicinoid receptor. Of equal importance, the observation that this pretreatment eliminated the delayed antinociception (at 2 days) emphasizes that the delayed effects depend upon events that occurred during the first hour after the civamide treatment. Finally, the absence of a reversal when the capsazepine was delivered at 2 days emphasizes that the antinociception at that time did not depend upon continued occupancy of the capsaicinoid receptor (a possibility given the lack of information on the actual tissue kinetics of these agents).

These results thus suggest that there are at least two components to the observed antinociceptive actions of capsaicinoids in general, and civamide in particular: (i) an acute occupancy-dependent desensitization; and (ii) a delayed, but persistent and occupancy-independent change in function that is generated by the initial effect of the civamide. The mechanism of acute desensitization may relate to depletion of transmitter stores, or maintained depolarization of the terminals. Alternately, it may reflect a post-synaptic action, such as the internalization of receptors (Mantyh et al., 1995). The mechanisms of the long-term changes are not known. With repeated exposure to capsaicin at higher concentrations, non-specific changes in terminal function may be induced. Calcium-dependent and -independent inactivation processes and osmotic changes due to the accumulation of intracellular ions may contribute (Bevan and Szolcsanyi, 1990; Holzer, 1991).

4.5. Civamide as an analgesic

Civamide at the highest dose (200 mg/kg) employed in this study, showed 14% morbidity. The cause of death appeared to be due to respiratory effects, most likely bronchial constriction, when this agent gained direct access to the airway. Such bronchial constriction is a well-known side-effect of this class of agents (Holzer, 1991). An increase in SP/CGRP levels in trachea could be a sign of stimulation of sensory afferents in this tissue. In general, based on our previous experiences with systemic capsaicin treatment in adult rats, we consider that the magnitude of the behavior, such as agitation, of even the highest dose of civamide was surprisingly minimal.

We conclude that civamide is an orally active analgesic agent, attenuating inflammation-induced hyperalgesia, acute thermal nociception and neuropathic pain. The antinociceptive effects of civamide are apparently mediated through an action on a vanilloid (capsaicin) receptor. The site of action of this agent on sensory afferent nerves is not clear. The decrease of CGRP in DRG neurons and sciatic nerves suggests that civamide targets the peptidergic afferent neurons. The rapid onset of the effects of this agent and no reduction in the peptide levels in dorsal spinal cord may suggest that the analgesic action of civamide is primarily a result of desensitization at the afferent terminals. This desensitization may be a reflection of impairment of normal functions of the peptide-containing sensory neurons, such as terminal release or the receptor inactivation.

Acknowledgements

This work was supported by GenDerm Corporation, and the Anesthesiology Foundation, Department of Anesthesiology, University of California, San Diego. We gratefully recognize the expert technical assistance of Fran Simonet-Magnuson for radioimmunoassay.

References

Bevan, S., Hothi, S., Hughes, G.A., James, I.F., Rang, H.P., Shah, K., Walpole, C.S.J. and Yeats, J.C., Development of competitive antagonist for the sensory neurone excitant, capsaicin. Br. J. Pharmacol., 102 (1991) 77.

Bevan, S. and Szolcsanyi, J., Sensory neuron-specific actions of capsaicin: mechanisms and applications, Trends Pharmacol., 11 (1990) 330–333.
Brand, L., Berman, E., Schwen, R., Loonians, M., Janusz, J., Bohne, R., Maddin, C., Gardner, J., Lahann, T., Farmer, R., Jones, L., Chiabrando, C. and Fanelli, R., NE-19550: a novel, orally active anti-inflammatory analgesic, Drugs Exp. Clin. Res., 13 (1987) 259–265.
Chaplan, S.R., Bach, F.W., Pogrel, J.W., Chung, J.M. and Yaksh, T.L., Quantitative assessment of tactile allodynia in the rat paw, J. Neurosci. Meth., 53 (1994) 55–63.
Dickenson, A., Ashwood, N., Sullivan, A.F., James, I. and Dray, A., Anti-nociception produced by capsaicin: spinal or peripheral mechanism?, Eur. J. Pharmacol., 187 (1990a) 225–233.
Dickenson, A., Hughes, C., Rueff, A. and Dray, A., A spinal mechanism of action is involved in the antinociception produced by the capsaicin analogue NE 19550 (olvanil), Pain, 43 (1990b) 353–362.
Dickenson, A.H., A cure for wind up: NMDA receptor antagonists as potential analgesics, Trends Pharmacol., 11 (1990) 307–309.
Dray, A., Campbell, E.A., Hughes, G.A., Patel, I.A., Perkins, M.N., Rang, H.P., Rueff, A., Seno, N., Urban, L. and Walpole, C.S.J., Antagonism of capsaicin-induced activation of C-fibres by a selective capsaicin antagonist capsazepine, Br. J. Pharmacol., 102 (1991) 78.
Fitzgerald, D.L. and Woolf, C.J., The time course and specificity of the changes in the behavioural and dorsal horn cell responses to noxious stimuli following peripheral nerve capsaicin treatment in the rat, Neuroscience, 9 (1982) 2051–2056.
Gamse, R., Capsaicin and nociception in the rat and mouse. Possible role of substance P, Naunyn Schmiedeberg's Arch. Pharmacol., 320 (1982) 205–216.
Gamse, R., Leeman, S.E., Holzer, P. and Lembeck, F., Differential effects of capsaicin on the content of somatostatin, substance P, and neurotensin in the nervous system of the rat, Naunyn Schmiedeberg's Arch. Pharmacol., 317 (1981) 140–148.
Go, V.L.W. and Yaksh, T.L., Release of substance P from the cat spinal cord, J. Physiol., 391 (1987) 141–167.
Hara, A., Sakurada, T., Sakurada, S., Matsumura, H. and Kisara, K., Anti-nociceptive effects of neonatal capsaicin in rats with adjuvant arthritis, Naunyn Schmiedeberg's Arch. Pharmacol., 326 (1984) 248–253.
Hargreaves, K., Dubner, R., Brown, F., Flores, C. and Joris, J., A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia, Pain, 32 (1988) 77–88.
Hastings, R.H. and Hua, X.-Y., Expression of calcitonin gene-related peptide by cultured rat alveolar type II cells, Am. J. Respir. Cell. Mol. Biol., 13 (1995) 563–569.
Hayes, A.G. and Tyers, M.B., Effects of capsaicin on nociceptive heat, pressure and chemical thresholds and on substance P levels in the rat, Brain Res., 189 (1980) 561–564.
Hayes, A.G., Oxford, A., Reynold, M., Shingler, A.H., Skingle, M., Smith, C. and Tyers, M.B., The effects of series of capsaicin analogues on nociception and body temperature in the rat, Life Sci., 34 (1984) 1241–1248.
Holzer, P., Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons, Pharmacol. Rev., 43 (1991) 143–201.
James, I.F., Walpole, C.S.J., Hixon, J., Wood, J.N. and Wrigglesworth, R., Long-lasting agonist activity produced by capsaicin-like photoaffinity probe, Mol. Pharmacol., 33 (1988) 643–649.
Jancso, G., Kiraly, E. and Jancso-Gabor, A., Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones, Nature, 270 (1977) 741–743.
Jhamandas, K., Yaksh, T.L., Harty, G., Szolcsanyi, J. and Go, V.L.W., Action of intrathecal capsaicin and its structure analogues on the content and release of spinal substance P: selectivity of action and relationship to analgesia, Brain Res., 306 (1984) 215–225.
Kantner, R., Kirby, M.L. and Goldstein, B.D., Increase in substance P in the dorsal horn during a chemogenic nociceptive stimulus, Brain Res., 338 (1985) 196–199.
Kim, S.H. and Chung, J.M., An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat, Pain, 50 (1992) 355–363.
Kim, S.H., Na, H.S., Sheen, K. and Chung, J.M., Effects of sympathectomy on a rat model of peripheral neuropathy, Pain, 55 (1993) 85–92.
Kim, Y.I., Na, H.S., Han, J.S. and Hong, S.K., Critical role of the capsaicin-sensitive nerve fibers in the development of the causalgic symptoms produced by transecting some but not all of the nerves innervating the rat tail, J. Neurosci., 15 (1995) 4133–4139.
Kuraishi, Y., Hirota, N., Sato, Y., Hanashima, N., Takagi, H. and Satoh, M., Stimulus specificity of peripherally evoked substance P release from the rabbit dorsal horn in situ, Neuroscience, 1989 (1989) 241–250.
Malmberg, A.B. and Yaksh, T.L., Antinociceptive action of spinal non-steroidal and antiinflammatory agents on the formalin test in the rat, J. Pharmacol. Exp. Ther., 263 (1992) 136–146.
Mantyh, P.W., DeMaster, E., Malhotra, A., Ghilardi, J.R., Rogers, S.D., Mantyh, C.R., Liu, H., Basbaum, A.I., Vigna, S.R. and Maggio, J.E., Receptor endocytosis and dendrite reshaping in spinal neurons after somatosensory stimulation, Science, 268 (1995) 1629–1632.
Meller, S.T., Gebhart, G.F. and Maves, T.J., Neonatal capsaicin treatment prevents the development of the thermal hyperalgesia produced in a model of neuropathic pain in the rat, Pain, 51 (1992) 317–321.
Nagy, J.I. and van der Kooy, D., Effects of neonatal capsaicin treatment on nociceptive thresholds in the rat, J. Neurosci., 3 (1983) 1145–1150.
Perkins, M.N. and Campbell, E.A., Capsazepine reversal of the antinociceptive action of capsaicin in vivo, Br. J. Pharmacol., 107 (1992) 329–333.
Schmauss, C. and Yaksh, T.L., In vivo studies on spinal opiate receptor systems mediating antinociception. II. Pharmacological profiles suggesting a differential association of mu, delta and kappa receptors with visceral chemical and cutaneous thermal stimuli in the rat, J. Pharmacol. Exp. Ther., 228 (1983) 1–12.
Shir, Y. and Seltzer, Z., A-Fibers mediate mechanical hyperalgesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of causalgiform pain disorders in rats, Neurosci. Lett., 115 (1990) 62–67.
Szallasi, A. and Blumberg, P.M., Resiniferatoxin and its analogs provide novel insights into the pharmacology of the vanilloid (capsaicin) receptor, Life Sci., 47 (1990) 1399–1408.
Szolcsanyi, J. and Jancso-Gabor, A., Sensory effects of capsaicin congeners. I. Relationship between chemical structure and pain-producing potency, Drug Res., 25 (1975) 1877–1881.
Szolcsanyi, J. and Jancso-Gabor, A., Sensory effects of capsaicin congeners. II. Importance of chemical structure and pungency in desensitizing activity of capsaicin-type compounds, Drug Res., 26 (1976) 33–37.
Watson, C.P.N., Tyler, K.L., Bickers, D.R., Millikan, L.E., Smith, S. and Coleman, E., A randomized vehicle-controlled trial of topical capsaicin in the treatment of postherpetic neuralgia, Clin. Ther., 15 (1993) 510–526.
Wheeler-Aceto, H., Porreca, F. and Cowan, A. The rat paw formalin test: comparison of noxious agents, Pain, 40 (1990) 229–238.
Winter, J., Bevan, S. and Campbell, E.A., Capsaicin and pain mechanisms, Br. J. Anaesth., 75 (1995) 157–168.
Wood, J.N., Walpole, C., James, I.F., Dray, A. and Coote, P.R., Immunochemical detection of photoaffinity-labelled capsaicin-binding proteins from sensory neurons, FEBS Lett., 269 (1990) 381–385.
Yaksh, T.L., Farb, D.H., Leeman, S.E. and Jessell, T.M., Intrathecal capsaicin depletes substance P in the spinal cord and produces prolonged thermal analgesia, Science, 206 (1979) 481–483.
Yaksh, T.L., Porgrel, J.W., Lee, Y.W. and Chaplan, S.R., Reversal of nerve ligation-induced allodynia by spinal alpha-2 adrenoceptor agonist, J. Pharmacol. Exp. Ther., 272 (1995) 207–214.
Yamamoto, T. and Yaksh, T.L., Stereospecific effects of a non-peptide NK1 selective antagonist, CP-96,345: antinociception in the absence of motor dysfunction, Life Sci., 49 (1991) 1955.

A PHASE 1 OPEN-LABEL, SINGLE-DOSE, DOSE ESCALATION STUDY IN HEALTHY SUBJECTS TO EVALUATE THE SAFETY AND PHARMACOKINETICS OF ORALLY ADMINISTERED CIVAMIDE (ZUCAPSAICIN)

Protocol No. WL-1001-03-01

IND No. 102,515

December 3, 2008 - Amendment 2

Winston Laboratories, Inc.
100 Fairway Drive, Suite 134
Vernon Hills, IL 60061

Phone: 847-362-8200
Fax: 847-549-9817

PROTOCOL AMENDMENT 2 APPROVAL

TITLE: A PHASE 1 OPEN-LABEL, SINGLE-DOSE, DOSE ESCALATION STUDY IN HEALTHY SUBJECTS TO EVALUATE THE SAFETY AND PHARMACOKINETICS OF ORALLY ADMINISTERED CIVAMIDE (ZUCAPSAICIN)

STUDY NO. WL-1001-03-01

VERSION: DECEMBER 3, 2008

SPONSOR: WINSTON LABORATORIES, INC.
100 FAIRWAY DRIVE, SUITE 134
VERNON HILLS, IL 60061

PHONE: 847-362-8200
FAX: 847-549-9817

PROTOCOL APPROVED BY:

SCOTT B. PHILLIPS, M.D.
Senior Vice President
Scientific Affairs

Date: 12/3/08

David A. Henninger, M.S., R.A.C.
Vice President
Operations

Date: 03-DEC-2008

TABLE OF CONTENTS

PAGE

Study Emergency Contact List

Winston Laboratories, Inc.			
Name	**Address**	**Phone/Fax**	**E-mail**
Sharon Sessoms Clinical Project Manager	Winston Laboratories, Inc 100 Fairway Drive, #134 Vernon Hills, IL 60061 USA	tel 847 362-8200 fax 847 362 8394 cell 847 494 7505	sharon@winstonlabs.com
Michael Menard, Ph.D. Director, Medical Affairs	Winston Laboratories, Inc. 100 Fairway Drive, #134 Vernon Hills, IL 60061 USA	tel 847 362-8200 fax 847 362-8394 cell 847 404-7101	michael@winstonlabs.com
Scott B. Phillips, M.D. Senior VP, Scientific Affairs	Winston Laboratories, Inc. 100 Fairway Drive, #134 Vernon Hills, IL 60061 USA	tel 847 362-8200 fax 847 362-8394 cell 312 371-7517	scott@winstonlabs.com
Heidi Fezatte Director, Clinical Compliance	Winston Laboratories, Inc. 100 Fairway Drive, #134 Vernon Hills, IL 60061 USA	tel 847 362-8200 fax 847 362-8394 cell 847 445-5260	heidi@winstonlabs.com

Protocol Synopsis

Study No.: WL-1001-03-01

Title: A Phase 1 Open-label, Single-Dose, Dose Escalation Study in Healthy Subjects to Evaluate the Safety and Pharmacokinetics of Orally Administered Civamide (Zucapsaicin)

Study Drug: Civamide liquid filled softgel capsules (5 mg)

Study Rationale: This will be the first in human study of the oral formulation of civamide. The main goals of this study are to determine if the oral formulation is well tolerated and bioavailable. Winston Laboratories has performed clinical studies using a nasal solution of civamide for headache and vasomotor rhinitis. Winston Laboratories has also performed clinical studies using a topical civamide formulation for osteoarthritis and psoriasis.

Sample Size: Up to 12 (6 per dose group) healthy, nonsmoking male or female subjects.

Objectives: Primary Objective: To determine the safety and tolerability of single, oral dose administration of civamide liquid filled softgel capsules in healthy subjects.

Secondary Objective: To characterize the pharmacokinetics of civamide following single, oral dose administration in healthy subjects.

Design: This is a Phase 1, open-label, single-dose, dose escalation first-time-in-human study of orally administered civamide in healthy subjects. Two ascending dosing cohorts, 5 mg and 10 mg, (N = 6 subjects per cohort) will be investigated

Table 1: Dose Escalation

Cohort	Dose	Regimen
1	5 mg	Oral administration of one 5 mg capsule (N=6) under fasted conditions
2a	5 mg	Oral administration of one 5 mg capsule (N=3) under fed conditions
2b	10 mg	Oral administration of two 5 mg capsules (N=3) under fed conditions

The Data Monitoring Committee (DMC) will review adverse events, vital signs, ECG, and data and clinical laboratory data collected from Day -1 to Day 7 for the 6 subjects in the 5 mg dose group (Cohort 1) prior to dose escalation to Cohort 2 ; the 5 mg and 10 mg cohort groups (2a and 2b). The DMC will include, at a minimum, the principal Investigator at the clinical site and the Sponsor's medical monitor.

The DMC will define dose limiting toxicities (DLT) as the following:

- Adverse Event Data:
  - AE (including clinically significant vital sign or ECG changes) of severe intensity or an SAE which are considered related to study drug by the DMC.
- Clinical Laboratory Data:
  - Increases or decreases from baseline considered clinically significant and related to study drug by the DMC and recorded as an AE of severe intensity. These also include changes in LFTs (AST, ALT, LDH) of $\geq$ 3 times the upper limit of normal or increases in serum creatinine of $\geq$ 0.5 mg/dl from the subject's Admittance (Day -1) value.

The following approach to dose escalation will be followed and monitored by the DMC:

In the event of any serious adverse event or DLT that in the investigator's opinion justifies termination or modification of the study, dosing will be stopped and the sponsor will be informed immediately. The DMC will then review the safety experience with the product, and decide whether the study must be terminated, whether dosing may resume at the same dose or at a lower dose, or whether the cohort must be enlarged. Adverse events and laboratory data from all patients of Cohort 1 will be reviewed by the DMC prior to dose escalation to Cohort 2 (2a and 2b).

Procedures: The study consists of a 21-day Screening Period (Day -21 to Day -2), a 5-day in-house study period (Day -1 to Day 4), and a final follow-up visit (Day 7 ± 1 day).

Screening (Day -21 – Day -2):

During the Screening Period, subjects will provide written informed consent and will then undergo screening procedures to determine study eligibility (inclusion/exclusion criteria, demographics, medical and medication history, exercise and dietary history, height, weight, and vital sign measurement, 12-lead electrocardiogram (ECG), clinical laboratory tests [urine drug and cotinine screen, blood alcohol screen, serology, and chemistry, hematology and urinalysis testing], and a complete physical examination). Follicle stimulating hormone (FSH) and serum HCG will be measured in all female subjects.

Admittance (Day -1):

Eligible subjects will be admitted to the research facility on Day -1 for the 4-night, 5-day in-house study period. The following procedures will be performed on Day -1 to confirm subject eligibility prior to dosing on Day 1: update medical and medication history, exercise and dietary history, measure vital signs, perform a 12-lead ECG, perform a complete physical examination, and collect blood and urine for clinical laboratory tests (urine drug and cotinine screen, blood alcohol screen, chemistry, hematology and urinalysis testing, and serum HCG in female subjects). Record any adverse events reported by the subject during the Screening Period. If confirmed eligible, subjects will be enrolled and dosed on Day 1.

Treatment Period (Days 1-4):

The following predose assessments will be performed on Day 1: adverse event (AE) and concomitant medication assessments and vital sign measurements. A blood

sample will also be collected for a predose pharmacokinetic analysis. After completion of all predose assessments, subjects will be dosed.

Safety will be assessed after dosing on Day 1. Adverse event and concomitant medications will be assessed from Day 1 to Day 4. Blood and urine for clinical laboratory testing will be performed 24 and 72 hours postdose. Vital sign measurements will be performed 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 48 and 72 hours postdose. A 12-lead ECG will be performed 2, 4, 6, 24, and 72 hours postdose. A complete physical examination will be performed approximately 72 hours postdose.

Pharmacokinetics will also be assessed relative to dosing on Day 1. Blood for pharmacokinetic analyses will be collected at pre-dose and at 10 minutes, 20 minutes, 0.5, 1, 2, 3, 4, 6, 12, 24, 36, 48, and 72 hours post-dose.

Subjects will be discharged from the clinical site after all 72-hour assessments are performed on Day 4.

Follow-Up Visit Day 7 (± 1 day):

Subjects will return to the clinic on Day 7 for a follow-up safety assessment. The following assessments will be performed on Day 7: adverse event (AE) and concomitant medication assessments, physical examination, vital sign measurements, clinical laboratories and 12-lead ECG.

Subject Population:

Inclusion Criteria

1. Subject voluntarily agrees to participate in this study and signs an IRB-approved informed consent prior to performing any of the screening procedures.
2. Healthy, determined by pre-study medical evaluation (medical history and physical examination, vital signs, ECG, and clinical laboratory evaluations).
3. Males or females between 18 to 45 years of age, inclusive.
4. Female subjects must be of nonchildbearing potential (surgically sterile [hysterectomy or bilateral tubal ligation] or post-menopausal $\geq$ 1 year) with follicle stimulating hormone [FSH] > 40 U/L).
5. Non-smokers (or other nicotine use) as determined by history (no nicotine use over the past year) and by urine cotinine concentration (< 200 ng/ml) at screening and/or Day -1.
6. Body mass index (BMI) between 18.5 and 30.5 kg/m^2, inclusive, at screening.
7. Subject is willing to eat a single high fat breakfast meal on Day 1 of the study.
8. Subject is willing and able to cooperate to the extent required by the protocol.

Exclusion Criteria

1. Clinically significant history or evidence of cardiovascular, respiratory, hepatic, renal, gastrointestinal, endocrine, neurological, immunological, or psychiatric disorder(s) as determined by the Investigator or designee.
2. Subjects with a history or clinical findings of coronary artery disease/cardiovascular disease or ECG findings judged clinically significant by the Investigator.
3. Any disorder that would interfere with the absorption, distribution, metabolism, or excretion of drugs.
4. Subjects with active upper gastrointestinal problems such as gastroesophageal reflux disease (GERD), or peptic ulcer disease.

5. Subject has known allergy or hypersensitivity to capsicum, Civamide, or capsaicin-containing products.
6. Positive screening test for Hepatitis B surface antigen, Hepatitis C antibody, or HIV antibody.
7. Subject has history of alcohol and/or illicit drug abuse within two years of entry.
8. Positive blood test for ethanol at screening or Day -1.
9. Positive urine drug test (cocaine, amphetamines, barbiturates, opiates, benzodiazepines, tetrahydrocannabinol [THC], etc.) at screening or Day -1.
10. Female subjects of childbearing potential or who are pregnant or breastfeeding.
11. Inability to refrain from consumption of coffee and caffeine containing beverages within 24 hours prior to Day -1 until discharge from the unit on Day 4.
12. Inability to refrain from use of alcohol or alcohol-containing foods, medications or beverages within 48 hours prior to Day -1 until discharge from the unit on Day 4.
13. Topical use of any capsaicin-containing product for 60 days prior to Day -1 until end of study participation.
14. Ingestion of any capsaicin-containing foods (capsicum, cayenne pepper, red pepper, green pepper, Scotch Bonnet, Habanero peppers, African chilies, Tabasco peppers, paprika, pimiento, Mexican chilies, Louisiana long pepper, Louisiana short pepper, Bird pepper, Garden pepper, Goat's pod, Grains of Paradise, Hot pepper, Hungarian Pepper, Ici Fructus, Sweet pepper, and Zanzibar pepper) for 48 hours before Day -1 until end of study participation.
15. Donation of blood (> 250 ml) or blood products within 2 months (56 days) prior to Day -1.

16. Consumption of grapefruit containing food/beverages or Seville oranges (orange marmalade) from 7 days prior to Day -1 until discharge from the unit on Day 4.
17. Use of over the counter (OTC) medications (including vitamins), prescription medications, or herbal remedies from the 14 days prior to Day -1 until discharge from the unit on Day 4. By exception, acetaminophen ≤ 1 gram per day is permitted and hormone replacement therapy is permitted.
18. Use of an investigational drug within 30 days prior to Day -1.
19. Unwilling to abstain from vigorous exercise from 48 hours prior to Day -1 until discharge from the unit on Day 4.
20. Subject has a history of lactose intolerance.

Safety Variables:

- Adverse Events.
- Laboratory Examinations (clinical chemistry, hematology, and urinalysis)
- Concomitant Medications.
- Vital Signs (sitting blood pressure, heart rate, respiratory rate, and body temperature).
- 12-lead ECG (QRS, RR, PR, QT, and QTc intervals). QTc will be determined using both Bazett and Fridericia correction methods.
- Complete physical examinations (assessment of general appearance and a review of systems (skin, eyes, ears, mouth, lymph nodes, respiratory, cardiovascular, gastrointestinal, musculoskeletal, and neurological systems).

Analytical Tests: Serum samples will be analyzed for serum civamide concentrations using a validated liquid chromatography mass spectrophotometry (LC/MS/MS) assay with a Lower Limit of Quantification (LLOQ) = 0.05 ng/mL.

Pharmacokinetic Analysis:

Because the low doses may not lead to quantifiable serum concentrations, the bioanalytical laboratory will be instructed first to analyze the 0 to 6 hour time point samples. If quantifiable serum concentrations are not seen by 6 hours postdose, then the remaining samples may not be analyzed. If quantifiable concentrations are seen, the remaining samples may be analyzed upon further instruction from the Sponsor. Therefore, full PK data analysis may not be possible on data from all subjects without quantifiable concentrations detected.

As data permit, non-compartmental analysis will be used to estimate the following pharmacokinetic parameters from the serum civamide concentration vs. actual time data:

- C_{max}: maximum serum civamide concentration determined directly from the concentration-time data.
- T_{max}: time to the maximum civamide concentration determined directly from the concentration-time data.
- AUC_{last}: Area under the serum civamide concentration time curve from time zero to the time of last quantifiable concentration (determined using the linear/log trapezoidal rule).
- Lambda z: The terminal elimination rate constant determined by selection of at least 3 data points on the terminal phase of the concentration-time curve.
- $T_{½}$: Terminal elimination half-life (ln2/lambda z).

- AUC_{inf}: Area under the serum civamide concentration time curve from time zero extrapolated to infinity (AUC_{last} + C_{last}/lambda z).
- $AUC_{\%extrap}$: Percent of AUC_{inf} that is due to extrapolation from T_{last} to infinity.
- Vz/F: Volume of distribution (Dose/AUC_{inf} *lambda z).
- CL/F: Total body clearance (Dose/ AUC_{inf}).

Statistical Analysis: Individual serum civamide concentration vs. time data will be listed and displayed graphically. Serum civamide concentration vs. time data will be summarized descriptively by dose group in tabular and graphical format. Civamide pharmacokinetic parameter data will be listed and summarized descriptively by dose group in tabular format. If data permit, regression analyses will be used to assess the dose proportionality of AUC_{last}, AUC_{inf}, and C_{max}, and the dose independence of $T_{½}$.

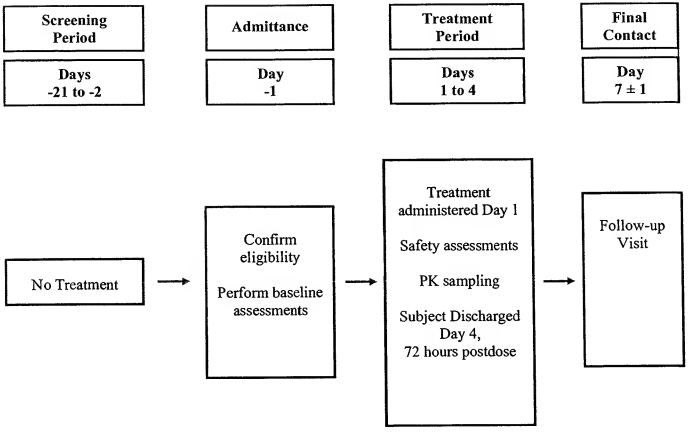
Safety data will be listed and summarized descriptively by dose group in tabular or graphical formats, as appropriate. Change from baseline may be calculated for select endpoints (blood pressure, heart rate, and QTc). Change from baseline data will be listed and summarized descriptively by dose group in tabular or graphical formats, as appropriate.

SCHEDULE OF EVENTS

	Screening	Admit	Treatment																		Follow-up
	Day -21 to -2	Day -1	Day 1													Day 2		Day 3	Day 4	Day 7 (±1)	
Procedure			Predose	10 Min	15 Min [c]	20 Min	0.5 hr	1	1.5	2	2.5	3	4	6	8	12	24	36	48	72	
Informed Consent	X																				
Demographics	X																				
Height/weight	X																				
Medical History	X	X																			
Dietary History	X	X																			
Concomitant meds	X	X																		X	X
Physical Exam	X	X														X				X	X
Clinical labs[a]	X	X																			
Urine drug and cotinine screen	X	X																			
Serum alcohol	X	X																			
FSH in females	X																				
Pregnancy test in females	X	X																			
12-lead ECG	X	X								X			X	X			X			X	X
Vital Signs[b]	X	X	X		X		X	X	X	X	X	X	X	X	X	X	X		X	X	X
Dosing			X (T_0)																		
Blood for PK			X	X		X	X	X		X		X	X	X		X	X	X	X	X	
AE monitoring	X																			X	
Discharge																					

a. Serum chemistry, hematology, urinalysis and serology.
b. Blood pressure, heart rate, respiratory rate, and body temperature.
c. Timepoint 15 minutes = 0.25 hr

STUDY FLOW CHART



Abbreviations and Definitions

AE	Adverse Event
ALT (SGPT)	Alanine Aminotransferase (serum glutamate pyruvate transaminase)
APD	Action-potential duration
APD_{50}	Action-potential duration at 50% repolarization
APD_{90}	Action-potential duration at 90% repolarization
AST (SGOT)	Aspartate Aminotransferase (serum glutamate oxalocacetate transaminase)
AUC	Area under the curve
$AUC_{0-\infty}$	Area under the curve from the time of dosing extrapolated to infinity
AUC_{0-t}	Area under the concentration-time curve from the time of dosing to the time of the last observation
BLQ	Below the Limit of Quantitation
BMI	Body Mass Index
bpm	Beats per minute
BUN	Blood Urea Nitrogen
CFR	Code of Federal Regulations (U.S.)
CGRP	Calcitonin gene-related peptide
CL/F	Total Body Clearance
cm	centimeter(s)
Cmax	Maximum plasma concentration
CPRU	Clinical pharmacology research unit
CRF	Case Report Form
DCF	Data Clarification Form
DLT	Dose Limiting Toxicity
DMC	Data Monitoring Committee
DMSO	Dimethyl sulfoxide
ECG	Electrocardiogram
FDA	Food and Drug Administration
FSH	Follicle Stimulating Hormone

GCPs	Good Clinical Practice Guidelines
GERD	Gastroesophageal Reflux
GI	Gastrointestinal
Gm	Gram
GRAS	Generally Regarded As Safe
HCG	β-Human Chorionic Gonadotropin
HEENT	Head, Eye, Ear, Nose, and Throat (including mouth and neck)
HIV	Human Immunodeficiency Virus
IB	Investigator's Brochure
ICH	International Conference on Harmonization
in	Inch(es)
IRB	Institutional Review Board
kg	Kilogram(s)
LC/MS/MS	Liquid Chromatography/Mass Spectrophotometry
lbs	Pounds
LDH	Lactic Dehydrogenase
LD_{50}	Median lethal dose
LLoQ	Lower limit of quantification
mg	Milligram
ml	Milliliter
mmHg	Millimeters of mercury
MTD	Maximum Tolerated Dose
ng	Nanogram
NOAEL	No Observed Adverse Effect Level
OTC	Over-the-counter
PK	Pharmacokinetics
RBC	Red blood cells
rpm	Respirations per minute
SAE	Serious Adverse Event
SDAR	Study Drug Accountability Record
SP	Substance P
t ½	Terminal elimination half-life
THC	Tetrahydrocannabinol

Tmax	Time to maximum serum concentration
VIP	Vasoactive intestinal peptide
Vz/F	Volume of Distribution
WBC	White blood cells
WLI	Winston Laboratories, Inc.

1.0 INTRODUCTION

1.1 Background Information

Civamide (Z-8-Methyl-N-vanillyl-6-nonenamide, cis-capsaicin, zucapsaicin), is the synthetically produced Z-isomer of naturally occurring capsaicin (E-8-Methyl-N-vanillyl-6-nonenamide). Capsaicin is the most active alkaloid found in hot red peppers from the plant genus Capsicum. Available data indicates that civamide is a TRPV-1 receptor modulator which acts by a mechanism similar to capsaicin.[1,2,3]

A brief review of the pharmacology and clinical effects of capsaicin, the E-isomer of civamide follows: TRPV-1 receptor modulators such as civamide and capsaicin can affect the synthesis, storage, transport, release or receptor binding of endogenous neuropeptides. Capsaicin, selectively depresses type-C nociceptive (pain) fibers and causes a release and subsequent desensitization to further release the neuropeptide, substance P (SP).[4,5] The result is an attenuation of the conduction and transmission of peripheral pain impulses. Since capsaicin is specific for type-C fibers, it relieves pain without affecting other more discriminatory senses, such as touch, pressure or vibration.

As demonstrated in both in vitro and in vivo models, capsaicin exerts its effects directly on receptor sites of the cell membrane in peripheral neurons[6] without intervention of a second-messenger system.[7,8,9] Initially, capsaicin-induced activation of the nonselective cation channels results in increased intracellular concentration of free Ca^{2+}.[6,7,8,9,10,11] The resulting influx of Ca^{2+}, which is independent of voltage-gated channels, causes depolarization of the neuron with subsequent release of neuropeptides.[9,12,13] Continued capsaicin application results in desensitization of the neuron to further stimuli.

1.1.1. CIVAMIDE NON-CLINICAL PHARMACOLOGY OVERVIEW

Pharmacologic studies were conducted to evaluate civamide's antinociceptive effect and effect on neuropeptide release and neuronal function when administered both orally and intrathecally.

Orally administered civamide (20-200mg/kg) was given to adult rats.[14] In the formalin test, civamide significantly suppressed the flinch response in a dose-dependent fashion (20-200mg/kg). This inhibitory effect started 1 hour after application, and was maintained for 7 days at the high dose. A competitive capsaicin antagonist, capsazepine (15 mg/kg, s.c.), which effectively blocks the TRPV-1 receptor, reversed the antinociceptive action of civamide (200mg/kg) on the formalin test when it was given either 5 minutes or 55 minutes after oral civamide administration. In contrast, capsazepine delivered 2 days after civamide had no effect upon the depressed formalin response. These results with capsazepine indicate that civamide's effects are mediated by the TRPV-1 receptor. Civamide produced a significant increase in the response latency on the thermal paw withdrawal test; the effect persisted for 2-3 days. A modest, but statistically significant increase in the threshold for paw withdrawal in rats with nerve ligation was reported using the Chung model for neuropathic pain. Civamide did not produce motor dysfunction at doses up to 200mg/kg. Neither calcitonin gene-related peptide (CGRP) nor SP concentrations in the dorsal or ventral spinal cord were altered by civamide (200mg/kg) whereas CGRP was modestly reduced 1 day post-dose in the dorsal root ganglia and sciatic nerves. These data suggest that civamide possessed analgesic activity with respect to several noxious stimuli, including inflammation-induced hyperalgesia, noxious thermal stimulation and nerve injury-induced tactile allodynia. The rapid onset and lack of change in the peptide levels in dorsal spinal cord suggests that the analgesic action of civamide is primarily a result of desensitization at the afferent terminals. This is probably mediated by at least two mechanisms: (i) an acute receptor occupancy dependent effect; and (ii) a persistent and receptor independent effect which is initiated by the acute exposure to the drug.

In a second study, a single intrathecal injection of either civamide (1 μg/20 μl, 10 μg/20 μl or 100 μg/20 μl), capsaicin (1 μg/20 μl, 10 μg/20 μl or 100 μg/20 μl), or vehicle, was given to male Sprague-Dawley rats (n=5/group).[1] Both civamide and capsaicin produced statistically significant dose-dependent decreases in the levels of SP and CGRP in the dorsal but not in the ventral horn of the rat spinal cord as compared to vehicle. For SP levels only, civamide

treatment produced a greater reduction than capsaicin at comparable doses. Depletion of CGRP was similar with both agents. Neither compound was shown to have an effect on vasoactive intestinal peptide (VIP). Thermal algesia, as measured by hot plate and tail flick tests, indicated that both civamide and capsaicin produced a statistically significant, dose-dependent increase in response latency. Civamide treatment (1 μg/20μl and above) produced a significantly greater response latency for the 49°C hot plate test than the same doses of capsaicin. No significant effect was noted for either compound for paw pinch threshold, indicating specificity for thermal but not mechanoreceptors. No evidence of motor dysfunction was observed.

In a third study, a single intrathecal injection of either civamide, synthetically produced capsaicin and naturally extracted capsaicin, or vehicle (20% DMSO) at doses of 5 μg/ml and 50 μg/ml was given to rats.[2] Statistically significant reductions in spinal cord SP levels were observed with both doses of civamide (5 and 50 μg/ml) as compared to vehicle. Although not statistically significant, greater reductions of spinal cord SP levels were observed in civamide-treated rats than in those treated with either synthetic or extracted capsaicin. No statistically significant differences existed between doses within each compound, or between active compounds, for SP depletion. In the hot plate latency test, both doses of civamide exhibited a statistically significant increase in response latency as compared to synthetic and extracted capsaicin at the same doses. Civamide had the overall greatest effect on reducing SP levels and increased the response latency when compared to synthetic capsaicin, extracted capsaicin and vehicle. None of the rats exhibited abnormal motor function.

1.1.2. Safety Pharmacology

The pharmacologic and toxicologic effects of long-term application of topical civamide cream were evaluated (incorrectly identified as capsaicin) at two concentrations (0.075% and 0.75%) as compared to vehicle to the sural nerve in adult Wistar rats (5/sex/group).[3] Civamide, at concentrations of 0.075% and 0.75% applied to rat hind limb twice daily for 10 weeks, produced no toxic effects on cell structure and no irreversible effects on the

function of peripheral nerve endings. In addition, no signs of dermal irritation or behavior changes were observed.

The cardiac electrophysiologic effects of civamide were evaluated in isolated Purkinje fibers. Using *in vitro* microelectrode techniques, civamide (10^{-5} M) shortened the action-potential duration at 50% repolarization (APD_{50}) from 193 ± 13 to 177 ± 12 ms ($p < 0.01$) and APD_{90} from 260 ± 15 to 248 ± 13 ms ($p < 0.01$) in isolated Purkinje fibers ($n = 10$). Nifedipine prevented the effects of civamide *in vitro*. These results show that civamide may shorten the APD of Purkinje fibers *in vitro*. The effects of civamide are prevented by preexposure of the Purkinje fibers to nifedipine, suggesting that the electrophysiologic effects of civamide may be mediated through blockade of calcium channels.[15]

A study was conducted to evaluate the potential cardiovascular and respiratory effects of civamide administered orally in capsules at doses of 1.5, 2.5, and 4mg/kg and placebo (empty capsule) in 8 beagle dogs (4 male / 4 female).[16] The dogs were administered the doses in ascending dose order for each phase of the testing. Each active dose was followed by a 3 to 8 day washout period, until each animal had received all treatments for both the cardiovascular and respiratory phases. Observations for mortality, morbidity, injury, and the availability of food and water were conducted twice daily for all animals. A detailed clinical examination of each animal was performed prior to dosing and following completion of the cardiovascular and respiratory monitoring periods. Body weights were measured and recorded on the day prior to each administration.

Blood samples were collected from all animals prior to dosing and at approximately 30 minutes, 1 hour, 2 hours, 4 hours and 6 hours after dosing, on each dosing occasion during the respiratory phase of the study, for determination of the plasma concentrations of the test article. The bioanalytical results of these samples are not yet available.

All animals survived until termination of the study. The unaudited draft report indicated that civamide, administered orally, via capsule, to male and female dogs at dose levels of 1.5, 2.5,

and 4 mg/kg did not produce any effects on mortality, body weight, body temperature, blood pressure, or any of the ECG or respiratory parameters tested. Salivation and emesis were noted following all test article treatments and, to a lesser extent, following the control (empty) capsule treatment. Based on these findings, it was concluded that oral administration of civamide produced no effects on cardiovascular or pulmonary parameters in dogs at doses up to and including 4 mg/kg.

Additional *in vivo* information on the effects of civamide on the cardiovascular system can be obtained from the minipig and dog studies conducted by Winston. In the 9-month chronic dermal minipig study[17], civamide cream (0.075, 0.75, 3.75%) was topically applied three times per day. There were no systemic effects at any dose. Electrocardiographic (ECG) examinations were conducted pre-study and at Week 1, 4.5 months, and 9 months. All ECGs were normal at all timepoints.

These data indicate that civamide did not adversely affect the cardiovascular, pulmonary, or nervous system.

1.1.3 CIVAMIDE ORAL TOXICOLOGY OVERVIEW

Single-dose acute oral civamide toxicity studies have been conducted in Swiss Webster mice (60.0 and 87.4 mg/kg) and Sprague-Dawley rats (female: 60 mg/kg; male: 90 mg/kg). The acute oral LD_{50} in mice was greater than 87.5 mg/kg in males and less than 60 mg/kg in females[18,19] In rats, the acute oral LD_{50} was greater than 90 mg/kg for males and greater than 60 mg/kg in females. Clinical signs in the surviving animals in both species included irregular respiration, piloerection, hunched posture and/or lethargy. Gross necropsy findings in both decedent and survivors were unremarkable.[20]

A two-week study in rats[21] designed as a dose-ranging investigation used doses of 250, 500, 750, and 1000 mg/kg/day. Mortality was noted at all doses: 250 mg/kg/day (1F), 500 mg/kg/day (1M), 750 mg/kg/day (1M, 4F) and 1000 mg/kg/day (5M, 3F). Deaths were considered to be related to test article reflux and aspiration based on gross and microscopic

evaluations of the lungs which revealed red discolored lungs and pulmonary congestion, hemorrhage, perivascular edema and emphysema. Clinical signs noted at 500 mg/kg/day and above included decreased activity and salivation. Increased absolute and relative liver weights were seen in females at 500 mg/kg/day and above.

In a four-week oral toxicity study of civamide in rats,[22] doses of 50, 250, and 500 mg/kg/day were administered. Mortality occurred at the mid- and high-doses: (2M and 2F at 250 mg/kg/day and 7M and 4F at 500 mg/kg/day). An internal investigation conducted at the laboratory revealed that these deaths were due to gavage errors and were unrelated to civamide; this was supported by microscopic examination which revealed hemorrhage, congestion and emphysema in the animals dying on study. Because of these deaths, an insufficient number of high-dose animals was available at study termination for histopathological examinations. Salivation was observed at all doses and hematology changes (e.g. decreased leukocytes, lymphocytes, and red blood cell parameters) occurred in the males at 500 mg/kg/day and in the females at 50 mg/kg/day and above. Alkaline phosphatase was increased in the high-dose females. Liver hypertrophy and vacuolation were noted at 250 and 500 mg/kg/day, but these are not adverse. Only the liver was evaluated microscopically in the low- and mid-dose animals. Toxicokinetic analyses were not performed as part of this study. A NOAEL was not established in this study.

An escalating dose study was conducted in two dogs[23] as follows: 50 mg/kg/day for 4 days, 100 mg/kg/day for 4 days, drug holiday for 4 days, 5 mg/kg/day for 4 days, 10 mg/kg/day for 4 days, 25 mg/kg/day for 4 days and 40 mg/kg/day for 4 days. Civamide was administered in capsules. Emesis occurred at all doses with the greatest severity at 40 mg/kg/day and above.

In a 4-week oral toxicity study,[24] 4 dogs/sex/dose were administered civamide in capsules at 3, 10, and 30 mg/kg/day. All animals survived, but emesis occurred on every dosing day at 3 mg/kg/day and above. Weight gain was reduced in the treated males and food consumption was slightly decreased in the high-dose females. Histopathology revealed a dose-related increase in the incidence and/or severity of pulmonary inflammation was observed in the

civamide-treated animals. This finding was associated with the emesis noted in the civamide-treated dogs and was not a systemic effect. There were no other test article-related effects. Toxicokinetic analyses were not performed as part of this study.

An embryo-fetal development study was performed in rats.[25] Civamide was administered orally via gavage at doses of 0, 5, 25, and 75 mg/kg/day on gestation days 6 through 17 at a final volume of 1 mL/kg/day. Deaths occurred in all groups; most of these were due to gavage errors as confirmed by the presence of red discolored lungs, esophageal perforation, or frothy liquid in the trache at necropsy. However, at 75 mg/kg/day, 5 deaths were considered to be test article-related. Clinical signs were noted at 25 and 75 mg/kg/day and included decreased activity, labored breathing and convulsions (high-dose only). There were no effects on body weight or food consumption. Civamide had no effect on mean numbers of implantation sites, corpora lutea, resorptions, postimplantation loss, viable fetuses, and fetal weight. There were no skeletal or visceral malformations; a single fetus from a high-dose animal had an external malformation. Civamide was not teratogenic.

1.1.4. Civamide Non-Clinical Pharmacokinetic Overview

An ADME study[26] of civamide (97.3 mg/kg and 474 mg/kg) in rats following oral gavage of radiolabeled drug has been performed. At least 28% of a single oral dose was absorbed. The highest concentrations of radiolabel were found in the liver, GI tract, bone marrow and kidney. Radioactivity in tissues was similar between dose groups indicating no dose-related accumulation of civamide in tissues. Males and females metabolized civamide differently, 5-12 radiolabeled metabolites were found in the urine depending on dose, sex and time of sample collection. The highest levels of radioactivity were excreted in the first 24 hours post-dosing. When the sexes were combined, approximately 83% of the 97.3 mg/kg dose and 68% of the 474 mg/kg dose were eliminated in the first 24 hours. Less than 1% of the total administered dose remained in the tissues and residual carcass for either dose group after 72 hours. No significant differences were noted between the high and low dose groups or between males and females with respect to route and rate of excretion.

A study was conducted to compare the toxicokinetics of civamide (10 and 50 mg/kg) following a single administration to rats via oral and dermal routes of administration.[27] Following dosing, plasma samples were collected at various time points over the next 24 hours and analyzed for civamide concentration levels. Systemic exposure to civamide increased linearly as the dermal dose increased from 10 to 50 mg/kg (AUC_{all} 10 mg/kg dermal = 146.03 hrs x ng/ml and AUC_{all} 50mg/kg dermal = 678.97 hrs x ng/ml). This was not true for the oral dose where non-linearity was seen (AUC_{all} 10 mg/kg oral = 45.19 hrs x ng/ml and AUC_{all} 50mg/kg oral = 110.90 hrs x ng/ml). Thus, the bioavailability of civamide by dermal administration was four to five times greater than seen by oral administration.

In vitro inhibition studies using pooled human liver microsomes were conducted to determine possible inhibitory effects of civamide from 0.01 to 100 µM on 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 P450 enzymes.[28] P450 isozyme identification was conducted at final civamide concentrations of 1, 10, and 25 µM. Expressed isozymes were used to identify which P450 enzymes were involved in the metabolism of civamide by monitoring the 4 identified metabolites and the loss of parent. It was determined that the P450 isozymes that appear most involved in the metabolism of civamide and the formation of the 4 identified metabolites are 2C19, 1A2, and 2C9. It was also noted that 3A4 appears to mediate some metabolism of civamide determined by the loss of parent without a proportional increase in the identified metabolite peak areas as observed with the other isozymes tested. The number of isozymes involved in the metabolism of civamide reduces the significance of any possible impact from individuals with P450 polymorphisms.

1.1.5 CIVAMIDE CLINICAL OVERVIEW

Two pharmacokinetic and fourteen (14) Phase 1 and 2 studies have been performed using intranasal or topical civamide formulations. Two Phase 1 studies examined the safety and tolerance of intranasal civamide in healthy adults. Safety and efficacy of intranasal civamide were examined in 3 additional double-blind Phase 2 studies of migraine, cluster headache, or

vasomotor rhinitis. Nine (9) studies examined the safety and/or effectiveness of topical civamide cream: 7 Phase 1 safety studies and 2 Phase 2 safety and efficacy studies in osteoarthritis.

One pivotal Phase 3 study and one open-label long-term safety study were completed examining the safety and efficacy of topical civamide in osteoarthritis. Two Phase 3 studies were completed examining the safety and efficacy of intranasal civamide for episodic cluster headache.

Common side effects of civamide nasal solution in humans include nasal stinging or burning, coughing, throat irritation, throat burning, sneezing, rhinorrhea, lacrimation and conjunctival injection. All known side effects of short-term use are transient and self-limiting. Risks associated with long-term use of civamide nasal solution have not yet been determined.

Side effects associated with the use of topical civamide cream in humans include stinging or burning, erythema and pruritus at the site of application. Civamide cream has been evaluated in a year-long study of chronic use. These local side effects are also expected with civamide patch. Any possible systemic effects of oral civamide or absorbed civamide from the patch formulation are not known at this time.

1.2 Study Rationale

Winston Laboratories, Inc. (WLI) is developing civamide for a variety of indications which involve oral, intranasal and dermal delivery routes. As outlined above, Phase 1, 2, and 3 clinical studies have been performed using the intranasal and dermal formulations of civamide. This protocol describes the first human study planned for the oral formulation of civamide. The main goal of this study is to determine if the oral formulation is well tolerated and bioavailable.

The planned civamide doses for use in this study are 5 and 10 mg. The 5 mg starting dose was selected based on the no observed adverse effect level (NOAEL) identified in the 28-day dog

study[24] and the 28-day rat study[22] for starting dose calculation purposes.[6] The lowest dose tested in the dog study was 3 mg/kg. Although emesis was observed at the lowest dose, dogs are known to be uniquely sensitive to gastric irritants and the pulmonary findings are likely due to aspiration of minor amounts of vomitus in the airways. Because these effects are not systemic in nature and it is unknown whether the emesis seen in dogs will occur in humans, the 3 mg/kg dose is considered the NOAEL for starting dose calculation purposes. The 5 mg starting dose provides a 19-fold safety margin based on a 60 kg human. For the 28-day rat study,[22] 500 mg/kg is considered the NOAEL for starting dose calculation purposes, which affords a 243-fold safety margin when starting with 5 mg in humans. Thus, the 5 mg starting dose does not pose unacceptable risks to human subjects and will be administered first in this study. For subsequent doses, the plan is to double the dose for each ascending cohort after a safety review on data from the previous cohort. The 10 mg dose is currently the highest dose planned for oral administration in this study.

1.3 COMPLIANCE WITH REGULATIONS AND LAWS

This study will be conducted in compliance with the protocol, the U.S. Food and Drug Administration Code of Federal Regulations (CFR), International Conference on Harmonization (ICH), Good Clinical Practice Guidelines (GCPs), and applicable regulatory requirements. This protocol complies with the ethical principles enunciated in the Declaration of Helsinki (Version 1989). Voluntary consent will be obtained from all eligible subjects prior to the performance of any protocol procedures.

2.0 OBJECTIVES

The primary objective of the study is to determine the safety and tolerability of single, oral dose administration of civamide liquid filled softgel capsules in healthy subjects.

The secondary objective is to characterize the pharmacokinetics of civamide following single, oral dose administration in healthy subjects.

3.0 STUDY DESIGN

This is a Phase 1, open-label, single-dose, dose escalation first-time-in-human study of orally administered civamide in healthy subjects. Cohort 1 received a 5mg dose in a fasted state. Because of gastric symptoms reported by subjects dosed in the fasted state in Cohort 1, Cohort 2 will have 3 subjects receiving a 5 mg dose in the fed state (Cohort 2a) and 3 more subjects at the next higher dose of 10 mg in the fed state (Cohort 2b) on Day 1 to complete Cohort 2.

Table 1: Dose Escalation

Cohort	Dose	Regimen
1	5 mg	Oral administration of one 5 mg capsule (N=6) in a fasted state
2a	5 mg	Oral administration of one 5 mg capsule (N=3) in a fed state
2b	10 mg	Oral administration of two 5 mg capsules (N=3) in a fed state

The Data Monitoring Committee (DMC) will review adverse event data and clinical laboratory data collected from Day -1 to Day 7 for the 5 mg dose group (Cohort 1) prior to approving dose escalation to the 10 mg cohort group (Cohort 2). The DMC will include, at a minimum, the principal Investigator at the clinical site and the Sponsor's medical monitor.

The DMC will define dose limiting toxicities (DLT) as the following:

- Adverse Event Data:
  - AE (including clinically significant vital sign or ECG changes) of severe intensity or an SAE which are considered related to study drug by the DMC.

- Clinical Laboratory Data:
  - Increases or decreases from baseline considered clinically significant and related to study drug by the DMC and recorded as an AE of severe

intensity. These also include changes in LFTs (AST, ALT, LDH) of ≥ 3 times the upper limit of normal or increases in serum creatinine of ≥ 0.5 mg/dl from the subject's Admittance (Day -1) value.

The following approach to dose escalation will be followed and monitored by the DMC:

In the event of any serious adverse event or DLT that in the investigator's opinion justifies termination or modification of the study, dosing will be stopped and the sponsor will be informed immediately. The DMC will then review the safety experience with the product, and decide whether the study must be terminated, whether dosing may resume at the same dose or at a lower dose, or whether the cohort must be enlarged. Adverse events and laboratory data from all patients of Cohort 1 will be reviewed by the DMC prior to dose escalation to Cohort 2.

Study procedures are outlined below. Please refer to the Schedule of Events on page 15 and Sections 7.0 and 8.0 for more detail.

3.1 SCREENING PERIOD (DAYS -21 TO -2)

The *Screening Period* will begin no more than 21 days (Days -21 to -2) prior to Day -1 of the *Treatment Period.* During the *Screening Period*, subjects will provide written informed consent and will then undergo screening procedures to determine study eligibility including: demographics, medical and medication history, height, weight, and vital sign measurements, 12 lead ECG, clinical laboratory tests (urine drug and cotinine screen, blood alcohol screen, serology, and chemistry, hematology and urinalysis testing), a brief diet history and a complete physical examination. Follicle stimulating hormone (FSH) and serum β-Human Chorionic Gonadotropin (HCG) will be measured in all female subjects. Subjects will be instructed not to donate blood or blood products during the *Screening Period* and through Day 4 of the study. Subjects will be instructed to avoid capsaicin-containing products for 48 hours prior to Day -1 and through Day 4 of the study. Alcohol and alcohol-containing foods and ingestion of caffeine are prohibited within 48 and 24 hours, respectively, prior to Day -1 and through Day 4 of the study. Foods or beverages containing grapefruit or Seville oranges are prohibited from 7 days prior to Day -1 through discharge on Day 4. Use of over-the-counter

(OTC; including vitamins) and prescription medications or herbal remedies is prohibited from 14 days prior to Day -1 through discharge on Day 4. Subjects should abstain from vigorous exercise from 48 hours prior to Day-1 until discharge from the study site on Day 4. Adverse events, concurrent medications and/or therapies will be updated throughout the screening period.

3.2 ADMITTANCE (DAY -1)

Subjects will be admitted to the research facility on Day -1. Subject eligibility will be re-evaluated including: updating of medical history, physical examination, vital sign measurement, 12 lead ECG, clinical laboratory tests, (urine drug and cotinine screen, blood alcohol screen, and chemistry, hematology and urinalysis testing), prior and concurrent medications and/or therapies, and a brief diet history. Female subjects will be given a serum pregnancy test. If confirmed eligible, subjects will be enrolled and dosed on Day 1. Adverse events, concurrent medications and/or therapies since Screening Period will be updated.

3.3 TREATMENT PERIOD (DAY 1 TO DAY 4)

During the *Treatment Period*, the subject's vital signs will be obtained prior to dosing. The single dose of civamide will be administered on Day 1. Adverse events, concurrent medications and/or therapies will be reviewed throughout the *Treatment Period*.

On Day 1 through Day 4, serial blood samples for PK analysis will be collected. On Day 1, the first sample will be a pre-treatment sample collected prior to administration of the dose. Then samples will be collected at 10 minutes, 20 minutes, 0.5, 1, 2, 3, 4, 6, 12, 24, 36, 48, and 72 hours postdose.

Safety will be assessed after dosing on Day 1. Adverse event and concomitant medications will be assessed from Day 1 to Day 4. Blood and urine for clinical laboratory testing will be performed 24 and 72 hours postdose. Vital sign measurements will be performed 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 48 and 72 hours postdose. A 12-lead ECG will be performed 2,

4, 6, 24, and 72 hours postdose. A complete physical examination will be performed approximately 72 hours postdose.

Blood draws for clinical laboratory measurements will be collected in the fasted state. Standard meals will be provided at regular times for breakfast, lunch, and dinner while confined in the CPRU with the exception of the breakfast meal served on Day 1. Breakfast will be withheld for Subjects in Cohort 1 on Day 1.. Subjects will fast starting at 22:00 each evening (Day -1 to Day 3). Snacks will also be provided as needed. Subjects in Cohort 2 will receive breakfast within 30 minutes prior to dose on Day 1. The breakfast will consist of a high fat meal with a caloric count of approximately 800-1,000 calories. Exercise is not permitted through Day 4 of the study. Time of meals (lunch, dinner and snacks) will be recorded on Day 1 in the source documents. The breakfast menu on Day 1 for Cohort 2 will be recorded in the source documents.

On Day 4, safety assessments will be performed and the subjects will be discharged from the clinical pharmacology research unit (CPRU) following the blood draw (72 hours).

3.4 Follow-up Visit (Day 7 ± 1)

Subjects will return to the clinic on Day 7 for a follow-up safety assessment. The following assessments will be performed on Day 7: adverse event (AE) and concomitant medication assessments physical examination, vital sign measurements, clinical laboratories and 12-lead ECG.

4.0 MATERIALS AND SUPPLIES

4.1 Study Drug Supplies

Study drug will be provided by WLI in the form of 5 mg liquid filled softgel capsules (see *Appendix C, Study Drug Packaging, Labeling, and Storage*).

4.1.1 Study Drug Packaging, Labeling, and Storage

a) Study Drug Labeling

The following information will be printed or recorded on the study drug labels: protocol number, instructions for study staff, drug name, quantity, drug strength, cautionary statement, "New Drug – Limited by U.S. Federal Law to Investigational Use", storage information, Sponsor's name and address.

b) Study Drug Storage

All study drug will be stored in tightly closed bottles at room temperature between 59° - 86° Fahrenheit (15° - 30° Celsius) in a locked cabinet from the time of receipt by the CPRU and throughout the study. Access to and dispensing of study drug supplies will be restricted to study personnel qualified to dispense study drug.

4.1.2 Study Drug Accountability and Subject ID#s

The CPRU will be responsible for recording complete and accurate study drug accountability information on the Study Drug Accountability Record (SDAR) by recording the date the study drug was received and the quantity received. At the end of the study, all study drug will be counted and returned by the site to WLI or designee will be recorded on the SDAR.

Each subject will be assigned a three-digit Subject ID# according to their presentation to the research facility and confirmation of eligibility on Day -1 (i.e. the first subject will be assigned Subject ID# 101). Subjects who withdraw for reasons other than adverse events may be replaced.

Cohort	Subject Numbers	Replacement Subject Numbers
1	101 - 106	1101 - 1106
2	201 - 206	2201 - 2206

4.1.3 Study Drug Administration

Dosing will take place after a 10 hour minimum overnight fast from food and drink other than water for subjects in Cohort 1. For subjects in Cohort 2, dosing will take place following the administration of a high fat meal. Oral civamide will be administered within 30 minutes after the start of the meal for subjects in Cohort 2 (2a and 2b). Administration of all doses will be conducted by qualified study personnel in the CPRU. Subjects will receive a single oral dose of one 5 mg capsule administered under fasted conditions in Cohort 1 or in Cohort 2, subjects will receive either a 5 mg or two 5 mg capsules (TDD = 10 mg) administered under fed conditions. The study staff will administer the dose orally with approximately 240 ml (8 ounces) of room temperature water for subjects in Cohort 1 and for subjects in Cohort 2 the dose will be co-administered with approximately 180 ml (6 ounces) of room temperature milk. The milk is being given to Cohort 2 to help ameliorate the gastric symptoms that were reported following dosing in Cohort 1. Study staff will confirm that study subjects consumed all doses administered by a visual hand and mouth check immediately after dosing.

Subjects must remain in an upright position for 2 hours postdose. Subjects in Cohort 1 will remain fasting until the 4 hour time point after which a standard lunch will be served.

4.2 MEALS AND SNACKS

Standard breakfast, lunch, dinner, and snacks will be provided to each subject at regular hours on Days -1 through 4 with the following exceptions: Breakfast will not be served on Day 1 for subjects in Cohort 1. A high fat breakfast will be served on Day 1 to subjects in Cohort 2 (2a and 2b)). For Cohort 2, breakfast will be served within 30 minutes prior to dosing. Subjects will be expected to complete the breakfast in 30 minutes or less. Study staff should counsel subjects to eat this entire meal. Study staff should perform a "clean plate" check at the end of the meal. Any uneaten food from this breakfast will be recorded in source documents. The timing of the start and finish of breakfast on Day 1 should also be recorded in the source documents. This high fat meal is being given to Cohort 2 to help ameliorate the gastric symptoms that were reported following dosing in Cohort 1. Breakfast will be provided to all subjects on Day 4 before the subject is discharged from the research facility. Time of meals for all subjects will be recorded on Day 1 in source documents.

5.0 SUBJECT SELECTION, WITHDRAWAL, AND COMPLIANCE

5.1 SUBJECT SELECTION CRITERIA

Up to 12 healthy subjects are planned for this study. Subjects will be recruited from the general population and invited to attend a screening visit to determine eligibility. Eligibility will be determined based on the inclusion/exclusion criteria listed below.

5.1.1 Inclusion Criteria

A subject may be included in the study if he/she meets all of the following inclusion criteria:

1. Subject voluntarily agrees to participate in this study and signs an IRB-approved informed consent prior to performing any of the screening procedures.
2. Healthy, determined by pre-study medical evaluation (medical history and physical examination, vital signs, ECG, and clinical laboratory evaluations).
3. Males or females between 18 to 45 years of age, inclusive.
4. Female subjects must be of nonchildbearing potential (surgically sterile [hysterectomy or bilateral tubal ligation] or post-menopausal $\geq$ 1 year) with follicle stimulating hormone [FSH] > 40 U/L).
5. Non-smokers (or other nicotine use) as determined by history (no nicotine use over the past year) and by urine cotinine concentration (< 200 ng/ml) at screening and Day -1.
6. Body mass index (BMI) between 18.5 and 30.5 kg/m2, inclusive, at screening.
7. Subject is willing to eat a single high fat breakfast meal on Day 1 of the study.
8. Subject is willing and able to cooperate to the extent required by the protocol.

5.1.2 Exclusion Criteria

A subject will not be included if he/she meets one or more of the following exclusion criteria:

1. Clinically significant history or evidence of cardiovascular, respiratory, hepatic, renal, gastrointestinal, endocrine, neurological, immunological, or psychiatric disorder(s) as determined by the Investigator or designee.
2. Subjects with a history or clinical findings of coronary artery disease/cardiovascular disease or ECG findings judged clinically significant by the Investigator.

3. Any disorder that would interfere with the absorption, distribution, metabolism, or excretion of drugs.
4. Subjects with active upper gastrointestinal problems such as gastroesophageal reflux disease (GERD), or peptic ulcer disease.
5. Subject has known allergy or hypersensitivity to capsicum, Civamide, or capsaicin-containing products.
6. Positive screening test for Hepatitis B surface antigen, Hepatitis C antibody, or HIV antibody.
7. Subject has history of alcohol and/or illicit drug abuse within two years of entry.
8. Positive blood test for ethanol at screening or Day -1.
9. Positive urine drug test (cocaine, amphetamines, barbiturates, opiates, benzo-diazepines, tetrahydrocannabinol [THC], etc.) at screening or Day -1.
10. Female subjects of childbearing potential or who are pregnant or breastfeeding.
11. Inability to refrain from consumption of coffee and caffeine containing beverages within 24 hours prior to Day -1 until discharge from the unit on Day 4.
12. Inability to refrain from use of alcohol or alcohol-containing foods, medications or beverages within 48 hours prior to Day -1 until discharge from the unit on Day 4.
13. Topical use of any capsaicin-containing product for 60 days prior to Day -1 until end of study participation.
14. Ingestion of any capsaicin-containing foods (capsicum, cayenne pepper, red pepper, green pepper, Scotch Bonnet, Habanero peppers, African chilies, Tabasco peppers, paprika, pimiento, Mexican chilies, Louisiana long pepper, Louisiana short pepper, Bird pepper, Garden pepper, Goat's pod, Grains of Paradise, Hot pepper, Hungarian Pepper, Ici Fructus, Sweet pepper, and Zanzibar pepper) for 48 hours before Day -1 until end of study participation.
15. Donation of blood (> 250 ml) or blood products within 2 months (56 days) prior to Day -1.
16. Consumption of grapefruit containing food/beverages or Seville oranges (orange marmalade) from 7 days prior to Day -1 until discharge from the unit on Day 4.

17. Use of over the counter (OTC) medications (including vitamins), prescription medications, or herbal remedies from the 14 days prior to Day -1 discharge from the unit on Day 4. By exception, acetaminophen ≤ 1 gram per day is permitted and hormone replacement therapy is permitted.
18. Use of an investigational drug within 30 days prior to Day -1.
19. Unwilling to abstain from vigorous exercise from 48 hours prior to Day -1 until discharge from the unit on Day 4.
20. Subject has a history of lactose intolerance.

5.2 Subject Withdrawal

The subject may withdraw consent from the study at any time without prejudice, and the Investigator may withdraw a subject from the study if deemed in the subject's best interest by the Investigator (see *Appendix A, Adverse Events, Administrative Requirements and Procedures* for detailed reasons why a subject may be withdrawn from the study).

Any subject withdrawing from the study during the *Treatment Period* should have all end-of-study or final procedures performed and recorded. Any subject who withdraws due to an adverse event after the first dose of study drug will be monitored until the signs and symptoms have either resolved or stabilized. Subjects who withdraw due to adverse event will not be replaced.

5.3 Subject Compliance

The research staff will make sure that the subject is compliant with protocol procedures, including but not limited to compliance with scheduled visits, refraining from exercise, and compliance with diet regimen provided.

6.0 TREATMENT, EXERCISE, AND DIET RESTRICTIONS

6.1 Prior Medications and/or Therapies

All prescription and OTC medications (including vitamins and herbal supplements) taken within 30 days of the *Screening Period* will be recorded. Use of OTC medications (including vitamins), prescription medications, or herbal remedies is not permitted from the 14 days prior to Day -1 until discharge from the unit on Day 4.

6.2 CONCURRENT MEDICATIONS AND/OR THERAPIES

It is anticipated that subjects enrolled into this study will be healthy and medication-free through Day 4 of the study. Hormone replacement therapy is permitted for post-menopausal women (non-childbearing potential). Acetaminophen ≤ 1 gram per day is permitted. (*Section 5.1, Exclusion Criterion #17.*)

6.3 DIETARY RESTRICTIONS

Coffee- and caffeine-containing beverages or medications are prohibited from 24 hours prior to Day -1 until discharge from the unit on Day 4. Tobacco- and nicotine-containing products are restricted for one year prior to the *Screening Period* (Day -21) and through Day 4 of the study. Alcohol-containing foods, medications, or beverages are restricted from 48 hours prior to Day -1 until discharge from the unit on Day 4. Breakfast will be restricted on Day 1 for subjects in Cohort 1. A high fat breakfast will be served prior to dosing on Day 1 to subjects in Cohort 2. See Section 4.2, Meals and Snacks for details.

Subjects must not consume capsaicin-containing foods (capsicum, cayenne pepper, red pepper, green pepper, Scotch Bonnet, Habanero peppers, African chilies, Tabasco peppers, paprika, pimiento, Mexican chilies, Louisiana long pepper, Louisiana short pepper, Bird pepper, Garden pepper, Goat's pod, Grains of Paradise, Hot pepper, Hungarian Pepper, Ici Fructus, Sweet pepper, and Zanzibar pepper) from 48 hours before Day -1 until end of study participation.

Consumption of grapefruit containing food/beverages or Seville oranges (orange marmalade) is prohibited from 7 days prior to Day -1 until discharge from the unit on Day 4.

6.4 EXERCISE REGIMEN

During the Screening Period, subjects will be informed that they must abstain from their usual daily exercise habits starting 48 hours prior to Day -1 through Day 4 of the study.

7.0 MEASUREMENTS AND EVALUATIONS

The following tests and evaluations will be performed at specified time points during the study after a signed and dated informed consent has been obtained from the subject. The specifics of each test or evaluation are detailed below and should be performed as specified. All results must be included in the subject's medical and study records.

7.1 DEMOGRAPHIC DATA

The Investigator/designee will record the subject's birth date, gender, race/ethnic origin, BMI, height, and weight during the Screening Period.

7.2 MEDICAL HISTORY

Medical history (indicating positive or negative history) will be recorded by the Investigator/designee during the *Screening Period* which will include the review of the following systems: cardiovascular, dermatologic, endocrine, gastrointestinal, genitourinary, head/eyes/ears/nose/throat (including mouth and neck) (HEENT), hematologic, hepatic, lymphatic, musculoskeletal, neurologic, psychiatric, renal, and respiratory. Surgical and reproductive history will also be recorded. A description (including date of onset, if known) is required for each positive response. An allergy history (including medications and food), substance abuse history, history of caffeine, alcohol and tobacco (nicotine) use will also be recorded. Medical history will be updated at admission on Day -1.

A dietary history will be obtained during screening and upon admission on Day -1 to ensure that subjects have not consumed the restricted food substances (i.e., capsaicin-containing foods, grapefruit containing food/beverages or Seville oranges) (*reference Section 6.3*).

7.3 PRIOR AND CONCURRENT MEDICATIONS

Prior and current medication use (including prescription, OTC and herbal products) from 30 days prior to screening until Day 7 follow up visit must be recorded by the Investigator/designee in the study records.

7.4 PHYSICAL EXAMINATION

The Investigator will conduct a physical examination on all subjects during the Screening Period, on Day -1, at 72 hours postdose, and at the Day 7 follow-up visit and will indicate whether the following systems are normal or abnormal: general appearance, dermatologic, HEENT, thyroid, lymph nodes, extremities, respiratory, gastrointestinal, musculoskeletal, cardiovascular, psychiatric and neurological systems. Any abnormalities must be noted and an assessment as to clinical significance must be made.

7.5 SAFETY EVALUATIONS

7.5.1 Vital Signs

Vital signs will be obtained and recorded at the following times during the study: Screening Period, Admittance Day -1, Treatment Period at Day 1 predose, and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 48, 72 hours postdose, and at the Day 7 follow-up visit. Blood pressure measurements (mmHg) and heart rate (bpm) will be taken while the subject is relaxed in a sitting position for at least 3 minutes with the arm at heart level. Respiratory rate will be recorded as the number of respirations per minutes (rpm). Oral temperature will be recorded in degrees Celsius (°C).

7.5.2 12-Lead Electrocardiogram (ECG)

An electrocardiogram will be conducted after at least a 5 minute resting period during the Screening Period, Admittance Day -1, and during the Treatment Period Day 1 at 2, 4, 6, 24 and 72 hours postdose and at the Day 7 follow-up visit. Subject must have an ECG judged clinically acceptable by the Investigator on Day -1. Any abnormalities will be noted and an assessment as to clinical significance will be made.

7.5.3 Laboratory Examinations

Routine laboratory tests will be conducted during the Screening Period, Admittance Day -1, during the Treatment Period at 24 and 72 hours postdose, and at the Day 7 follow-up visit (see *Appendix D, Laboratory Specimen Collection, Management* and *Shipping* for details). Blood will be collected according to the procedures specified for the following clinical laboratory analyses.

a) Hematology (hemoglobin, hematocrit, white blood cell (WBC) count, red blood cell (RBC) count, and platelet count. The differential will analyze neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

b) Chemistry (sodium, potassium, chloride, calcium, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, creatinine, blood urea nitrogen (BUN), glucose, uric acid, total protein) and serum alcohol (*Screening Period* and Day -1)

c) Virology for Hepatitis B, Hepatitis C, and HIV (*Screening Period* only)

d) Females subjects will have specimen obtained for determination of FSH (*Screening Period* only).

e) Urinalysis (specific gravity, pH, color, blood, bilirubin, protein, glucose, and microscopic analysis).

f) Urine drug testing will be done to screen for drugs of abuse including the following: amphetamines, barbiturates, benzodiazepines, cocaine, opiates, phencyclidine, and cannabinoids. Cotinine testing will also be performed. These tests will be performed during the *Screening Period* and Day -1 only.

7.5.4 Pregnancy Tests

In addition to the clinical chemistry, a serum pregnancy test will be performed for female subjects from the sample collected during the *Screening Period.* Subjects with a positive serum pregnancy test will be excluded from the study. A serum pregnancy test will also be

conducted with the chemistry tests before dosing (Day -1) after the subject checks into the research facility.

7.6 PHARMACOKINETIC EVALUATIONS

7.6.1 Blood Sample Collection

A total of 14 blood samples, approximately 10 ml each, will be obtained from each subject during the study for pharmacokinetic evaluation (see *Appendix B, Acceptable Visit and Blood Sample Time Period Ranges*). Blood samples will either be drawn through an in-dwelling venous cannula, heparin lock, or by direct venipuncture. (The cannula and heparin lock may be replaced as necessary.) When obtaining samples through the in-dwelling cannula or heparin lock, the first 1.5 ml of blood will be discarded and the next approximately 10 ml collected in a 10 ml evacuated serum tube will be saved for analysis. Blood samples will be allowed to clot for 20 minutes, centrifuged at 2000 rpm (approximately 667 x g) for 15 minutes, and the serum separated and frozen at -70°C within 1 hour of collection. Samples will be shipped to the laboratory according to the instructions in *Appendix D, Laboratory Specimen Collection, Management, and Shipping.*

On Day 1 of the *Treatment Period*, 12-hour serial blood samples will be collected prior to study drug administration, and at 10 minutes, 20 minutes, 30 minutes, and 1, 2, 3, 4, 6, and 12 hours after dosing. On Day 2, samples will be obtained at 24 and 36 hours postdose. The 48 hour and 72 hour samples will be obtained on Days 3 and 4, respectively. Strict adherence to scheduled pK blood collection times must be followed, see acceptable ranges in Appendix B, Acceptable Visit and Blood Draw Time Period Ranges.

8.0 STUDY PROCEDURES

The following detailed procedures are performed at each clinic visit. All results will be documented on the subject's medical/research charts, source documents, and electronic database as required (see *Schedule of Events*).

8.1 SCREENING PERIOD DAY -21 THROUGH DAY -2

1. Obtain informed consent prior to performing any tests and procedures:
   - Ensure that the subject has read, understood, and voluntarily signed and dated the IRB-approved informed consent.
   - A completed copy of the informed consent will be given to the subject, and the original will be filed in the subject's research files.
2. Assess subject eligibility by reviewing the inclusion and exclusion criteria.
3. Obtain demographic data and medical history.
4. Review prior medications (prescription and OTC, including vitamins and herbal supplements) taken by the subject over the past 30 days and document concurrent medication use (see *Sections 6.1 and 6.2* for medication exclusions, restrictions, and allowances).
5. Obtain brief exercise regimen, diet history with particular emphasis on any capsaicin, alcohol, caffeine-containing foods or beverages and smoking (nicotine) history.
6. Obtain serum specimen for FSH and record results for female subjects only.
7. Perform physical examination and vital signs (blood pressure, heart rate, temperature, and respirations). Record height and weight.
8. Perform and record results of the electrocardiogram (ECG). ECG must be judged clinically acceptable by the Investigator.
9. Obtain clean catch urine sample for urinalysis, urine drug screen and cotinine.
10. Blood samples for chemistry and hematology tests, serology, serum alcohol and pregnancy test for female subjects. Pregnancy results must be negative prior to administering the first dose.
11. Instruct subjects to refrain from consumption of coffee- and caffeine-containing beverages within 24 hours prior to Day -1, alcohol or alcohol-containing foods, medications, or beverages, and capsaicin-containing foods 48 hours prior to Day -1.
12. Remind subjects to refrain from consuming grapefruit foods or juices and Seville oranges 7 days prior to Day -1.
13. Remind subjects to refrain from vigorous exercise 48 hours prior to Day -1.

14. Remind the subject not to donate blood or blood products while in the study.
15. Schedule and instruct the subject to report to the research facility on Day -1.

8.2 ADMITTANCE DAY -1

1. Review the selection criteria to ensure the subject remains eligible.
2. Update medical history, if necessary.
3. Perform and record results of the electrocardiogram (ECG). ECG must be judged clinically acceptable by the Investigator.
4. Obtain clean catch urine sample for urinalysis, drug screen and cotinine.
5. Blood sample for chemistry and hematology tests, serum alcohol and pregnancy test for female subjects.
6. Review medications taken by the subject and any changes in the subject's current medications since the last visit.
7. Review any changes in the subject's exercise and diet regimen.
8. If the subject is not eligible, discharge the subject from the study.
9. Perform physical examination and vital signs.
10. Record any adverse events reported by the subject during the Screening Period.

8.3 TREATMENT PERIOD DAY 1

1. Review the subject's concurrent medications.
2. Obtain vital signs (blood pressure, heart rate, temperature, and respirations): Day 1 predose, and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, and 12 hours postdose.
3. Collect the predose blood sample (10 ml) prior to administration of the study drug by inserting a cannula or heparin lock in one of the subject's arms. The cannula or heparin lock will be removed on Day 2 after the scheduled blood sample draw. Subsequent samples will be obtained by direct venipuncture. (Venipuncture may also be used if necessary prior to the 24 hour sample.)
4. Perform and record results of the electrocardiogram at 2, 4, and 6 hours postdose.
5. Record any adverse events reported by the subject or observed by the Investigator/designee during the day.

6. Collect serial blood samples (10 ml each) 10 minutes, 20 minutes, 30 minutes, 1, 2, 3, 4, 6, and 12 hours after dosing.
7. Remind the subject to refrain from exercising.

8.4 TREATMENT PERIOD DAY 2 – DAY 3

1. Obtain vital signs (blood pressure, heart rate, temperature, and respirations) at 24 hours postdose Day 2 and 48 hours postdose Day 3.
2. Review the subject's concurrent medications.
3. Collect the single blood sample (10 ml) at 24 and 36 hours postdose Day 2 and 48 hours postdose Day 3.
4. Record any adverse events reported by the subject or observed by the Investigator/designee during each day.
5. Blood sample for chemistry and hematology tests and clean catch urine sample for urinalysis at 24 hours postdose, Day 2.
6. Perform and record results of the electrocardiogram at 24 hours postdose, Day 2.
7. Remind the subject to refrain from exercising each day.

8.5 TREATMENT PERIOD DAY 4

1. Obtain vital signs (blood pressure, heart rate, temperature, and respirations), physical examination and 12 lead ECG at 72 hours postdose.
2. Review the subject's concurrent medications.
3. Perform venipuncture and collect the single pharmacokinetics blood sample (10 ml) 72 hours postdose.
4. Collect blood sample for chemistry and hematology tests and urine specimen for urinalysis.
5. Record any adverse events reported by the subject or observed by the Investigator/designee.
6. Examine subject's venipuncture site(s). Any clinically significant observations will be reported as adverse events.
7. Instruct the subject to be available for a follow-up visit on Day 7 ($\pm$ 1 day).

8. Discharge the subject from the research facility. If subject is discharged with an on-going AE, the Investigator may require the subject to return to the facility for follow-up assessments.

8.6 Post Treatment Period Day 5 and Day 6

The subject does not have to report to the research facility for any study procedures during the last two days of the 3-day *Post Treatment Period.* However, the subject has to take note of any new or changes in concurrent medications taken or adverse events experienced and report them during the follow-up visit, Day 7.

8.7 Follow-up Visit Day 7

1. Obtain vital signs (blood pressure, heart rate, temperature, and respirations), physical examination and 12 lead ECG at 7 days postdose.
2. Review the subject's concurrent medications.
3. Collect blood sample for chemistry and hematology tests and urine specimen for urinalysis.
4. Record any adverse events reported by the subject or observed by the Investigator/ designee.
5. Discharge the subject from the research facility. If subject is discharged with an on-going AE, any unresolved adverse events should be followed to resolution.

9.0 SAFETY VARIABLES

The following measurements and evaluations will be obtained for the assessment of safety and tolerance.

9.1 Adverse Events

Adverse events (AEs) will be collected throughout the study by direct interview of the subject. For each adverse event, the following information will be recorded on the subject's source document(s): onset date and time, end date and time, severity, duration, relationship to study

drug, action taken, and outcome. If the subject experiences a serious adverse event (SAE), the Investigator may discontinue the subject from study participation. The Investigator must notify the sponsor and IRB within 24 hours of receipt of the information. The Investigator will instruct the subject to notify the research facility should any adverse event occur within 3 ±1 days of discharge from the facility. (See definitions of an AE and SAE in *Appendix A, Adverse Events, Administrative Procedures, and Requirements*). Subjects who withdraw due to an adverse event will not be replaced.

9.2 VITAL SIGNS

Vital signs as described in *Section 7.5.1* will be performed during the *Screening Period (Day -21 to -2),* on Admittance, Day -1, during the Treatment Period, on Day 1 through Day 4, and at the Day 7 follow-up visit. Clinically significant measurements or changes in vital signs from baseline will be recorded as an AE.

9.3 LABORATORY EXAMINATIONS

Routine laboratory examinations as described in *Section 7.5.3* will be performed during the *Screening Period (Day -21 to -2),* on Admittance, Day -1, during the Treatment Period, on Day 2 and Day 4 prior to discharge from the facility, and at the Day 7 follow-up visit. Clinically significant abnormalities will be recorded as AEs and the study subject will be followed until the test(s) has (have) normalized or stabilized.

9.4 12 LEAD ELECTROCARDIOGRAMS

12 lead ECGs as described in Section 7.5.2 will be performed at the Screening Period (Day -21 to -2), on Admittance (Day -1), during the Treatment Period (Day 1) at 2, 4, and 6 hours postdose, on Day 2, on Day 4, prior to discharge from the facility, and at the Day 7 follow-up visit. An ECG judged clinically acceptable by the Investigator is required at Screening and Admittance Day -1. The ECG will be recorded at a paper speed of 25 mm/sec. The following ECG parameters will be collected: PR, RR, QRS intervals, QT interval, and QTcB interval (using Bazett's square root correction). QTcF will be calculated using Fridericia's cube root

correction. The ECG data will be evaluated for the presence of abnormalities. Clinically significant abnormalities will be recorded as AEs.

10.0 ANALYTICAL PROCEDURES

Serum samples will be analyzed for civamide by a validated liquid chromatography mass spectrophotometry (LC/MS/MS) assay[29] with a Lower Limit of Quantitation (LLOQ) of civamide of 0.05 ng/ml. Analysis of serum samples will be performed by MPI Research, Inc., State College, PA. (See *Appendix D, Laboratory Specimen Collection, Management, and Shipping).*

The bioanalytical laboratory will be instructed to analyze the 0 to 6 hour time point samples. If quantifiable serum concentrations are not seen by 6 hours postdose, then the remaining samples may not be analyzed. If quantifiable concentrations are seen, the remaining samples may be analyzed upon further instruction from the Sponsor. Therefore, full PK data analysis may not be possible on data from all subjects without quantifiable concentrations detected.

11.0 PHARMACOKINETIC AND STATISTICAL ANALYSIS

11.1 PHARMACOKINETIC ANALYSIS

As data permit, non-compartmental analysis will be used to estimate the following pharmacokinetic parameters from the civamide serum concentration vs. actual time data:

- C_{max}: maximum civamide serum concentration determined directly from the concentration-time data
- T_{max}: time to the maximum civamide concentration determined directly from the concentration-time data
- AUC_{last}: Area under the civamide serum concentration time curve from time zero to the time of last quantifiable concentration (determined using the linear/log trapezoidal rule).

- Lambda z: The terminal elimination rate constant determined by selection of at least 3 data points on the terminal phase of the concentration-time curve.
- $T_{½}$: Terminal elimination half-life (ln2/lambda z)
- AUC_{inf}: Area under the civamide serum concentration time curve from time zero extrapolated to infinity (AUC_{last} + C_{last}/lambda z)
- AUC%extrap: Percent of AUC_{inf} that is due to extrapolation from T_{last} to infinity
- Vz/F: Volume of distribution (Dose/AUC_{inf} *lambda z)
- CL/F: Total body clearance (Dose/ AUC_{inf})

If adequate numbers of males and females are enrolled in the study, gender differences in pharmacokinetic parameters of civamide will be evaluated.

Individual civamide serum concentration vs. time data will be listed and displayed graphically. Civamide serum concentration vs. time data will be summarized descriptively by dose group in tabular and graphical format. Civamide pharmacokinetic parameter data will be listed and summarized descriptively by dose group in tabular format. If data permit, regression analyses will be used to assess the dose proportionality of AUC_{last}, AUC_{inf}, and C_{max}, and the dose independence of $T_{½}$.

11.2 Safety Analysis

The incidence of adverse events (based upon the occurrence of at least one adverse event) will be the primary safety endpoint of the study. Secondary safety endpoints include the incidence of adverse events classified according to preferred term and body system.

Safety data will be listed and summarized descriptively by dose group in tabular or graphical formats, as appropriate. Change from baseline may be calculated for select endpoints (blood pressure, heart rate, and QTc). Change from baseline data will be listed and summarized descriptively by dose group in tabular or graphical formats, as appropriate.

12.0 DATA MANAGEMENT

12.1 ELECTRONIC DATA CAPTURE

An electronic data capture system (ClinBase™) has been developed and provided by PAREXEL for use in the clinical study. The Investigator will ensure the accuracy, completeness, and timeliness of the data reported to the Sponsor. Data collection processes and procedures will be reviewed and validated to ensure completeness, accuracy, reliability, and consistency. A complete audit trail will be maintained of all data changes. The Investigator or designee will cooperate with the Sponsor's representative(s) for the periodic review of study documents to ensure the accuracy and completeness of the data capture system at each scheduled monitoring visit.

Electronic consistency checks and manual review will be used to identify errors or inconsistencies. Errors or inconsistencies in study data will be identified and recorded on data clarification forms (DCF). After the resolution of any DCFs and the data cleaning process is complete, the database will be locked.

12.2 STUDY DOCUMENTS

The Investigator or designee will prepare and maintain adequate and accurate study documents (medical records, ECGs, adverse event and concomitant medication reporting, raw data collection forms, etc.) designed to record all observations and other pertinent data for each subject receiving study drug.

The Investigator will allow Sponsor representatives, contract designees, authorized regulatory authority inspectors, and the IRB to have direct access to all documents pertaining to the study.

13.0 QUALITY ASSURANCE, AUDITING, AND ARCHIVING OF STUDY DOCUMENTS

Quality Assurance review and auditing will take place at the discretion of WLI's Senior Vice-President of Scientific Affairs. The study will be monitored during the trial by WLI according to GCP guidelines. The study monitor will have access to all records necessary to ensure integrity of the data and will periodically review the progress of the study with the principal Investigator.

Frequent communication between the study site and the Sponsor is essential to ensure that the safety of the study is monitored adequately. The Investigator will make all appropriate safety assessments on an ongoing basis. The Sponsor's medical monitor may review safety information as it becomes available throughout the study.

It is the responsibility of the Investigator to maintain a comprehensive and centralized filing system of all relevant documentation. The Investigator will be instructed to retain all study records required by the Sponsor as well as the regulatory documents in a secure facility with limited access for the following required period: a minimum of two years after the last approval of a marketing application in an ICH region and until there are no pending or contemplated marketing applications in an ICH region, or after at least two years have elapsed since the formal discontinuation of clinical development of the investigational product. A copy of regulatory documents will be archived at WLI upon completion of the study.

14.0 INVESTIGATOR AGREEMENT

I have read and I understand Protocol No. WL-1001-03-01, entitled ***"A Phase 1 Open-label, Single-Dose, Dose Escalation Study in Healthy Subjects to Evaluate the Safety and Pharmacokinetics of Orally Administered Civamide (Zucapsaicin)"***, and agree to conduct the study as outlined herein. I also agree to treat the protocol as confidential information and restrict its use accordingly. Should I or any of my colleagues, desire to publish any portion of its unpublished material, I will obtain written authorization from Winston Laboratories, Inc.

I further agree to permit representatives of Winston Laboratories, Inc. and their designated representatives to perform trial related monitoring and auditing, including auditing of the IRB, and regulatory documents by providing direct access to all source data and documents.

_____ _____

Signature of Principal Investigator Date

Printed Name and Address of Principal Investigator

Shwe Gyaw, MD
PAREXEL International, Inc.
Baltimore CPRU
3001 South Hanover Street, 7th Floor
Baltimore, MD 21225